Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Optimization of screening methods for the evaluation of the antileishmanial potential of halophytes and macroalgae from the Iberian Coast

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Thesis to obtain the master's degree in Medical Parasitology

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‘’A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.’’

Marie Curie

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Abstract

Leishmaniases are parasitic diseases caused by *Leishmania* parasites, which are transmitted to mammals by the bite of sand flies. In the Mediterranean basin, *L. infantum* is the etiological agent of human visceral leishmaniasis, the most severe form of the disease. The obvious limitations of current available drugs still provide an undoubtedly motivation for research of novel compounds with antileishmanial potential. In the last decades, marine organisms or species living in a terrestrial habitat strongly influenced by the sea have been shown to be potential sources of rich bioactive compounds. Thus, the search for marine natural products with antileishmanial activity has gathered interest and, as a result, some promising extracts and compounds have been described. For assessment of the *in vitro* activity of natural extracts mainly axenic promastigotes and amastigotes have been used. Although the amastigote-macrophage system remains the ideal model, its use has been neglected mainly due to the lack of sensitive and rapid *in vitro* screening methods, which rely on the inherent difficulties of the intracellular stage. In this context, the main objective of this work is to contribute for the study of the antileishmanial potential of macroalgae and halophytes, through the optimization of screening methods focusing on the intracellular amastigote form. The direct counting method was optimized and used for the assessment of extracts activity against intracellular amastigotes. Concerning the parasite rescue assay, lysis conditions were established. However, aberrant results were obtained when the effect of amphotericin B and that of different infection ratios was assessed, in comparison to the direct counting method results. As the complete optimization of the parasite rescue assay was not possible and reminding the shortcomings of the direct counting method, halophyte and macroalgae extracts were tested against *L. infantum* promastigotes. Halophytic extracts were in general inactive against *L. infantum* promastigotes. *Spergularia rubra* and *Inula crithmoides* dichloromethane extracts were the most active, but were also extremely cytotoxic towards THP-1 macrophages. Concerning macroalgae, the hexane and dichloromethane extracts of *Cystoseira tamariscifolia* and the hexane extract of *C. usneoides* were highly active towards the promastigotes with IC_{50} values below 35 µg mL^{-1}, yet they had also poor selectivity. *C. baccata* hexane extract was the most selective algal extract (SI > 1.32). Additionally, *C. baccata* and *C. barbata* hexane:dichloromethane extracts, obtained by hot soxhlet extraction, displayed interesting antileishmanial activities (IC_{50}, 50.2 ± 2.6 and 15.1 ± 2.9, respectively) towards the intracellular stage of *L. infantum* combined with promising SI values (>2.5 and >8.3, respectively), reinforcing the main argument of this work that intracellular amastigotes are the ideal *in vitro* model to be used in drug screening purposes, which may yield a larger number of positive hits if implemented in future screening efforts.

Keywords: *L. infantum;* halophytes; macroalgae; resazurin; intracellular amastigotes

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List of Abbreviations

ACE	Acetone
AFU	Arbitrary fluorescence units
AmB	Amphotericin B
ANOVA	Analysis of variance
Ca^{2+}	Calcium ion
CanL	Canine leishmaniasis
CL	Cutaneous leishmaniasis
CO_2	Carbon dioxide
DCL	Diffuse cutaneous leishmaniasis
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Foetal bovine sérum
H_2Od	Distillated water
HEX	Hexane
HEX:DCM	Hexane:Dichloromethane
HIV	Human immunodeficiency virus
IC_{50}	Inhibitory concentration of 50% of individuals
IHMT	Instituto de Higiene e Medicina Tropical
MCL	Mucocutaneous leishmaniasis

MeOH	Methanol
MTT	3-[4,5-dimethylthiazol-2-y1]-2,5 diphenyl tetrazolium bromide
NO	Nitric oxide
NP	Natural product
PBS	Phosphate-buffered saline
Pen/Strep	Penicillin/Streptomycin
PKDL	Post-kala-azar dermal leishmaniasis
PMA	Phorbol myristate acetate
PNA	Negative peanut agglutinin
PNA^-	Infective parasites
PNA^+	Non-infective parasites
PS	Phosphatidylserine
ROS	Reactive oxygen species
RPMI	Liquid culture medium ‘’Roswell Park Memorial Institute’’
RT	Room temperature
Sb^v	Pentavalent antimonials
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SI	Selectivity index
SPSS	Statistical package for social sciences
VL	Visceral leishmaniasis

Chapter I – Introduction

1. Leishmaniases

Leishmaniases are parasitic diseases caused by intracellular protozoa parasites belonging to the *Leishmania* genus and transmitted to mammals by the bite of female phlebotomine sand flies. At least 20 *Leishmania* species are known as cause of disease in humans and approximately 98 sand flies species, from 800 known, are proven or suspected vectors of the human disease (Ezquerra, 2001; Maroli et al., 2013).

Transmission can be classified as zoonotic or anthroponotic, according to the main reservoirs. For example, *L. infantum*, in the Mediterranean Basin, causes zoonotic visceral leishmaniasis, hence the main reservoir is the domestic dog and humans are accidental hosts (Ruiz-Fons et al., 2013). *L. donovani*, in India, causes anthroponotic visceral leishmaniasis, where humans play the most important role in the transmission of the disease (Singh et al., 2006).

Concerning human leishmaniasis, these flagellates particularly affect mononuclear phagocytic cells and, depending on the parasite species and immune system of the host, the disease can have a wide range of clinical forms. However, three basic clinical forms of human leishmaniasis are recognized: cutaneous (CL), mucosal (MCL) and visceral (VL) leishmaniasis, the latter being also known as kala-azar (Desjeux, 2004; Rey, 2011).

With 350 million people at risk of contracting the disease, the overall prevalence of leishmaniasis is estimated to be 12 to 14 million people in tropical, subtropical and temperate regions of New and Old Worlds (WHO, 2010a; Ponte-Sucre et al., 2013). It occurs in 5 continents and is endemic in 98 countries and 3 territories (Alvar et al., 2012). This disease remains a severe public health problem in developed countries, being considered by World Health Organization as a neglected tropical disease in developing countries (Desjeux, 1996; WHO, 2010a).

Although human visceral leishmaniasis has been a notifiable disease in 40 countries since the 1950's, the number of cases actually occurring is clearly underestimated. This can be due to undiagnosed, misdiagnosed or unreported cases, discontinuous distribution within endemic areas, lack of diagnostic resources and of access to medical healthcare (Desjeux, 2004; Campino et al., 2006; WHO, 2010a).

In fact, in developing countries poverty and leishmaniasis seem to interact in a vicious cycle, where poor house holdings, lack of protective measures, mainly concerning

reservoir hosts (e.g., dogs), malnutrition and immunologic deficits, lack of resources and of access to health care leads to an increased risk of exposure to leishmaniasis, morbidity and mortality. Moreover, impoverished populations encounter many barriers for proper treatment of the disease caused by medical debt, disfigurement stigma, work problems and poor education. Therefore, its annual incidence is growing in the world's poorest countries (Alvar et al., 2012).

1.1. *Leishmania* parasite

In the beginning of the XIX century, Cunningham and Borovsky were the first to describe this parasite, however, Leishman, Donovan, Wright, Lindenberg and Vianna identified the parasite and indicated it as the etiological agent of leishmaniasis. In 1903, Ronald Ross, attributed the generic name of *Leishmania*. In 1904, Nicolle was the first to name the parasite *L. infantum.* Four years later described dogs as reservoirs in Tunis and was able to maintain an axenic culture in the laboratory (WHO, 2010).

Leishmania parasites belong to phylum Sarcomastigophora, class Zoomastigophora, order Kinetoplastida, family Trypanosomatidae and *Leishmania* genus. However, the classification of *Leishmania* species has been a controversial. Three different categories can be distinguished, according to their development pattern in vectors. More specifically, *Leishmania* species can be considered as hypopylaria if they multiply in the pylorus, ileum and rectum bowel of the vector, peripylaria if they proliferate in the hindgut or suprapylaria if they divide in the midgut and foregut. This theory, described by Lainson et al. (1977), was later supported by phylogenetic studies, and has been used until now (Fraga et al., 2010).

Concerning this classification, *Sauroleishmania* subgenus belong to the hypopylaria group and are considered to be nonpathogenic to mammals, as they apparently only infect Old World reptiles upon ingestion of infected phlebotomine sand flies. Conversely, in the *Viannia* subgenus, which includes two main complexes, namely *L. braziliensis* complex (*L. braziliensis*, *L. peruviana*) and *L. guyanensis* complex (*L. guyanensis*, *L. panamensis*), parasites are classified as peripylaria and pathogenic, as it is able to infect mammals as armadillos, sloths, anteaters and also dogs, being restricted to the New World (Bates, 2007; WHO, 2010).

Lastly, parasites of the *Leishmania* subgenus are found in the skin, viscera and blood of Old World and Neotropical mammals. Parasites from this subgenus belong to the suprapylaria group, which is divided in five main complexes: *L. donovani* complex [*L. donovani*, *L. infantum* (syn. *L. chagasi*)], *L. tropica* complex (*L. tropica)*, *L. major* complex (*L. major)*, *L. aethiopica* complex (*L. aethiopica)* and *L. mexicana* complex (*L. mexicana*, *L. amazonensis*, *L. venezuelensis*; WHO, 2010).

1.1.1. Morphology and Life Cycle

Leishmania parasites alternate between two morphologic forms (extracellular and intracellular) in their life cycle, a consequence of two physiological and biologically different environments. The extracellular form, promastigote, develops in the digestive tube of the invertebrate host, and the intracellular form, amastigote, in the vertebrate host cells of the mononuclear phagocytic system (Kamhawi, 2006).

Promastigotes have a slender body, about 9 to 15 µm long, multiply actively by longitudinal binary fission, and contain a single nucleus and a free flagellum, which confers mobility to the cell (Figure 1A,B). Furthermore, as all tripanosomatids, they possess a unique subcellular structure with mitochondrial DNA, namely kinetoplast (Shlomai, 2004).

When an infected female phlebotomine sand fly bites a mammal, it lacerates blood vessels during feeding and regurgitates parasites (Figure 2). Once in cutaneous capillaries, promastigotes are phagocytized by neutrophils, dendritic cells and macrophages, migrating into the phagolysosome and differentiating into the non-flagellated amastigote form. At this point, whether the infected macrophages stay at the inoculation site or spread the infection to other organs will determine the progress of disease. Oval amastigotes forms, about 5 µm by 3 µm, are obligate intracellular parasites with tropism to macrophages, where they multiply. These forms are afflagelate, with a large off-center nucleus, the kinetoplast and an axoneme extended to the edge of the parasite (Zeibig, 1997; Dedet and Pratlong, 2003; Figure 1B,C).

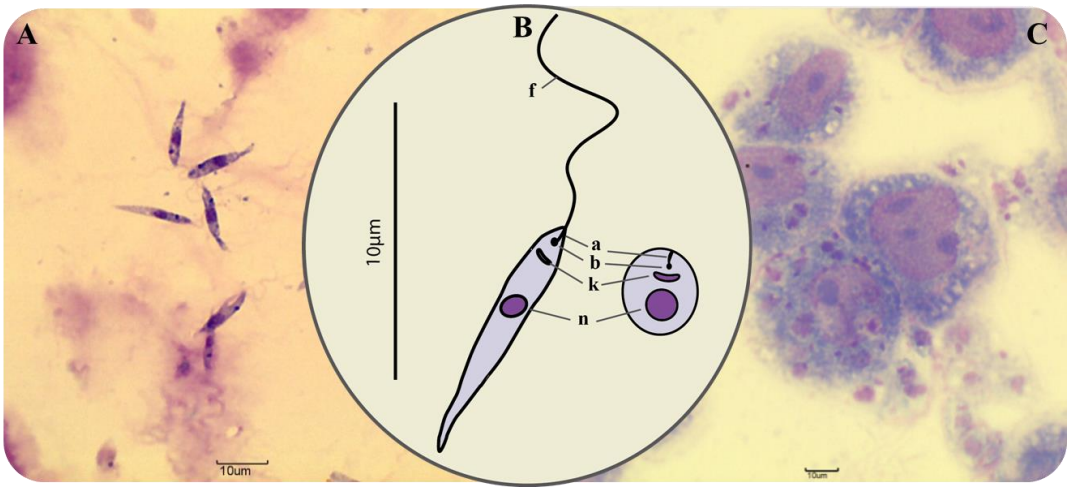

Figure 1 *L. infantum* life stages. **A**) Axenic promastigotes (x400). **B**) Illustrative morphology of promastigote (left) and amastigote (right) forms. **a** axoneme, **b** basal body, **f** flagellum, **k** kinetoplast, **n** nucleus. **C**) *in vitro* intracellular amastigotes inside THP-1 macrophages (x1000);

From the moment that parasites enter the bloodstream, neutrophils, natural killer, T and B cells work together, trying to eliminate the parasite, by inducing cytokines and chemokines production, complement-mediated lysis, nitric oxide (NO) production and other leishmanicidal factors. However, *Leishmania* has the capacity to evade the immune response by inducing the activity of suppressive cytokines such as IL-10, synthetizing antioxidants as superoxide dismutase and by down-regulating NADPH oxidase and iNOS expression, which will reduce or inhibit ROS and NO production (Shio and Olivier, 2010; Gupta et al., 2013). Moreover, *L. major* glycoconjugates such as lipophosphoglycan and metalloproteinase gp63 have been pointed as virulence factors for their role in resistance to complement mediated lysis (revised by Kamhawi, 2006).

The parasite multiplies within parasitophorous vacuoles and amastigotes are eventually released, when their quantity and damage to the host cell are significant to achieve the rupture of macrophages. Released amastigotes will reinvade other macrophages, increasing the number of infected cells and extending the infection in the mammalian host (Rey, 2011).

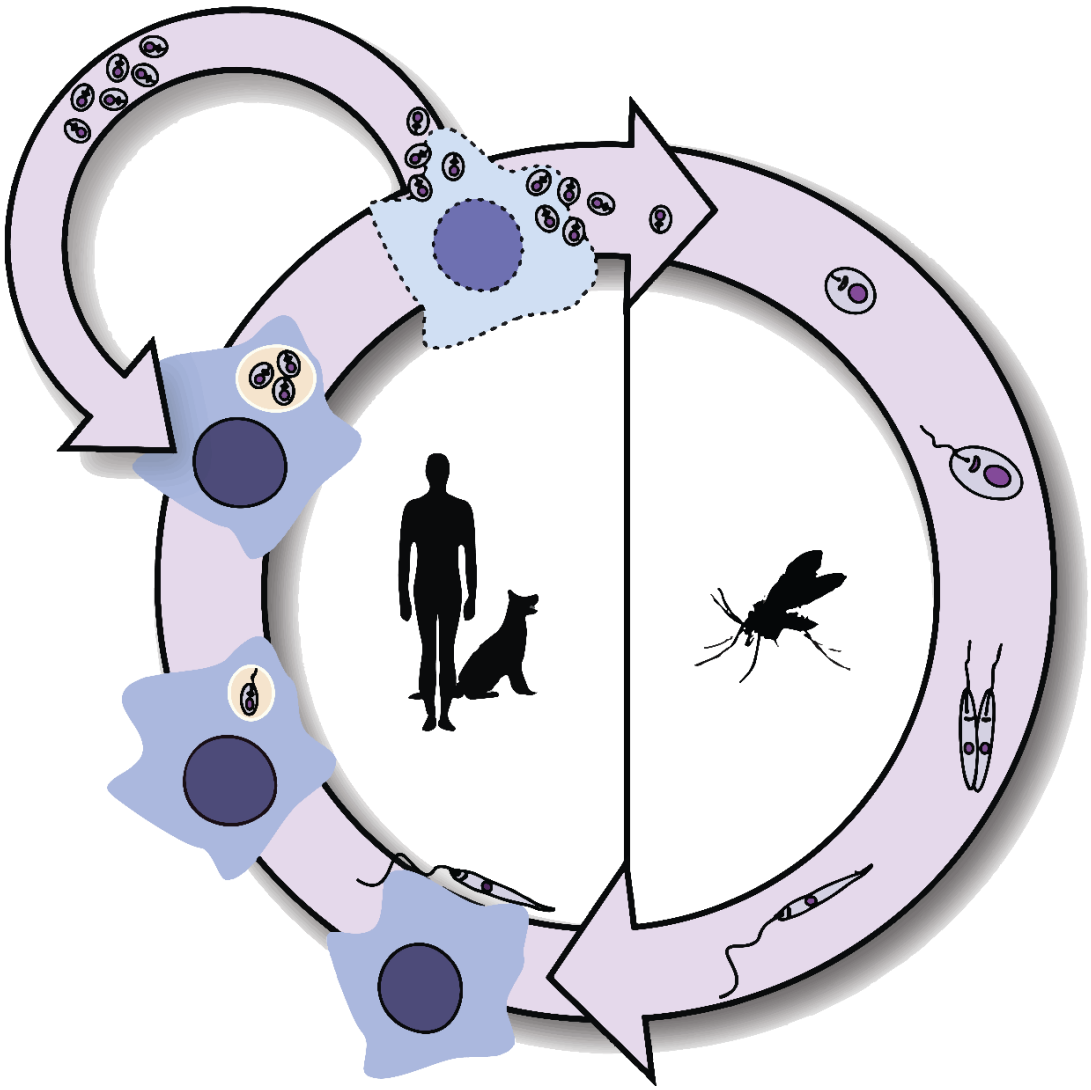

Figure 2 The life cycle of *Leishmania* spp. Inside the vector (right), parasites face the process of metacyclogenesis, differentiate into metacyclic promastigotes and migrate to the phlebotomine proboscis. When the sand fly bites, it regurgitates promastigotes into blood vessels of the mammalian host (left). Promastigotes infect mammalian cells and differentiate into oval amastigotes. Amastigotes multiply, lyse the cell and reinvade other cells. The phlebotomine sand fly takes a blood meal and ingests amastigotes completing and also restarting the cycle (De Assis et al., 2012; Original from Oliveira, M.).

When a phlebotomine sand fly takes a blood meal, amastigotes are ingested and will evolve and multiply in the vector digestive tube, where they transform into procyclic promastigotes (Figure 3; Ezquerra, 2001; Rey, 2011).

Inside the vector, metacyclogenesis begins with the transformation of amastigotes into small procyclic promastigotes with short flagella, which will further develop into larger, slender forms, called nectomonads, within 2-3 days (Figure 3).

At day 4, leptomonads are formed from nectomonads and by day 5-7 differentiate into metacyclic forms. Leptomonads produce a promastigote secretory gel (PSG) which will comprise the last promastigote forms mentioned, in the thoracic midgut. Metacyclic forms are small cells with an elongated flagellum, highly motile and are the infective stage to the mammalian host. The latter forms will accumulate at the stomodeal valve, being available for transmission. When a phlebotomine sandfly bites a mammalian host, the cycle restarts (Kamhawi, 2006).

All *Leishmania* species are morphologically indistinguishable, with high structural homogeneity in all stages of their biological life cycle. The differentiation of these parasites used to rely in extrinsic characteristics such as type and location of the primary lesions they cause in humans, type of hosts and geographic distribution (Bogitsh and Cheng, 1998; Ezquerra, 2001).

Nowadays, with the advances in molecular methods, *Leishmania* parasites are identified based in intrinsic characteristics, such as its genome, which allow the identification of the species, subspecies and variants.

Analysis of isoenzymes by electrophoresis (multilocus enzyme electrophoresis - MLEE) is currently the gold standard technique for species identification and genus taxonomy. Performed by only few reference laboratories, it has limitations as the requirement to isolate parasites in culture (Chargui et al., 2012). In this technique, strains with the same enzymatic profiles are grouped, creating a taxonomical unity called zymodeme. In the Mediterranean basin, *L. infantum* MON-1 is the principal zymodeme responsible for the majority of VL cases (Pratlong et al., 2004; Campino et al., 2006).

It has already been reported variations in the response for treatment by various species, emphasizing the importance of a correct species identification for the appropriate and effective treatment (Arevalo et al., 2007; Tiuman et al., 2011).

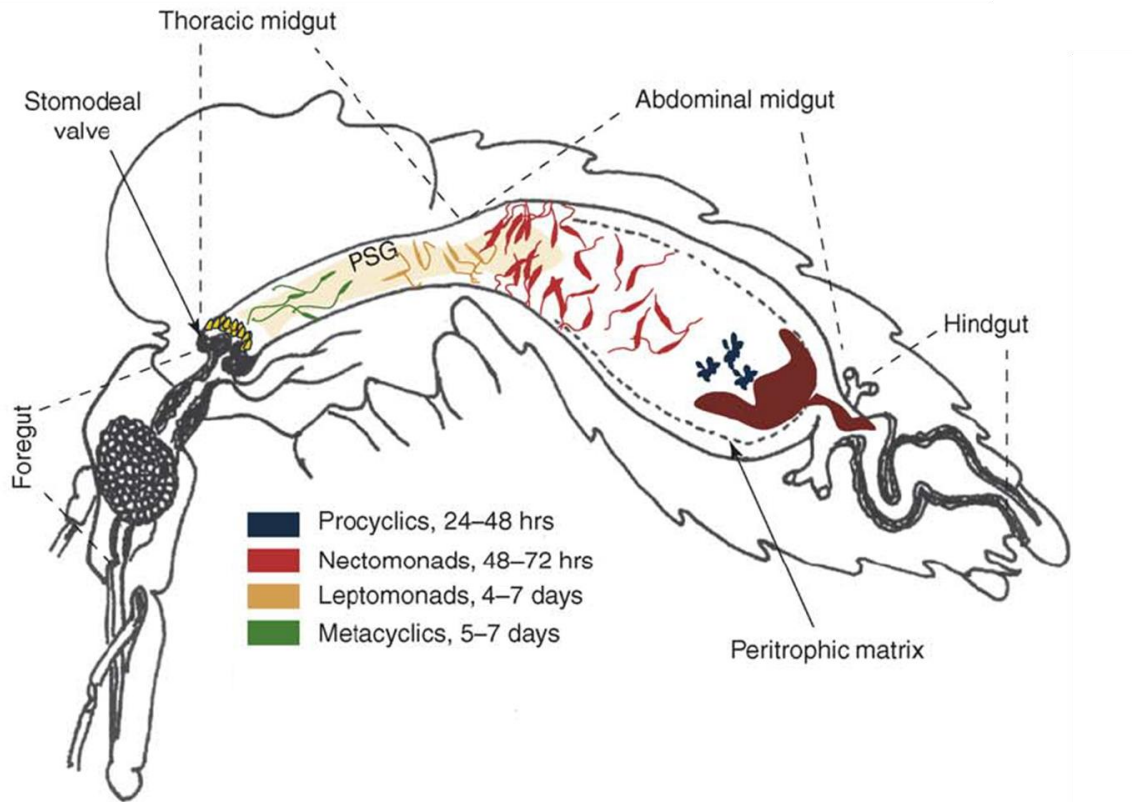

Figure 3 Schematic representation of the development of *Leishmania* parasites inside the phlebotomine sandfly vector (Adapted from Kamhawi, 2006).

1.2. Vectors and Reservoirs

Phlebotomine sand flies belong to class Insecta, subclass Pterygota, order Diptera, suborder Nematocera, family Psychodidae and subfamily Phlebotominae. They are small, have blade-shaped hairy wings and when at rest they are easily identifiable because of their pointed wings at an angle above the abdomen (Dedet and Pratlong, 2003).

Sand flies can be found in various habitats from dry regions as deserts and savannas to tropical rain forests. They prefer to rest during the day, at dark places such as henneries, rodent burrows, wall holes and cracks as well as human dwellings and become active at dusk and during the night. Only female phlebotomine sand flies are hematophagous, as they need blood to provide nutrients for the maturation of their eggs (Lane and Crosskey, 1993; Dedet and Pratlong, 2003; WHO, 2010).

The geographic distribution of leishmaniasis reflects the presence or absence of parasite species and their respective vector species. From five genera accepted there are two with epidemiologic and medical importance, which all the proven vectors of

leishmaniasis. They include: *Lutzomyia* genus, in the New World and *Phlebotomus* genus, vectors in the Old World (Dedet and Pratlong, 2003; Rey, 2011).

Although many authors mention that some species of the *Sergentomyia* genus have no medical importance some reports have questioned this dogma. *S. minuta* and *S. darling* were both found naturally infected with *L. major* in Portugal and Mali, respectively. These reports suggest that if its anthropophilic behavior is proved, these species may play a potential role in the transmission of leishmaniasis and probably other vector borne diseases (Campino et al., 2013).

The efficacy of parasite transmission by sand flies depends on intrinsic factors of the vector such as longevity, density, trophic preferences and exo/endophilic characteristics, the proximity of their preferential reservoirs and the responsiveness of human infection (Ezquerra, 2001).

In relation to *Leishmania*'s reservoirs, in the Mediterranean basin, Latin America and some parts of China, the domestic dog is the most important one. Concerning *L. infantum,* the dog is considered as the only primary reservoir of infection. Dogs seem to be an ideal reservoir, as they become regularly infected, often presenting high parasite loads, which favors parasite transmission. They also share the same environment with humans, favoring the maintenance of disease. Interestingly, the most common zymodeme of *L. infantum (*MON-1) is also predominantly isolated in dogs (Dantas-Torres, 2007; Diniz et al., 2008; Rey, 2011).

Besides the visceral manifestations, cutaneous lesions are the most common effects of canine leishmaniasis (CanL) that can be accompanied by loss of body weight and appetite, fever and diarrhea (Solano-Galego et al., 2011). Some accidental reservoirs have been referred in the literature such as horses (Rolão et al., 2005), goats, buffaloes and cows (Bhattarai et al., 2010) but recently, some authors have been reporting cases of leishmaniasis in domestic and peridomestic cats in Portugal (Maia et al., 2010), Italy (Poli et al., 2002), Brazil (Savani et al., 2004) and France (Ozon et al., 1998), emphasizing the potential role of cats as natural reservoirs and in the epidemiology of disease.

Besides domestic reservoirs wild animals as well as non-domestic canidae, rodents and hyraxes are the main wild reservoir hosts in the Old World while opossums, sloths, anteaters, non-domestic canidae and rodents are important reservoirs in the New World (WHO, 2010).

1.3. Epidemiology and clinical forms of the disease

The outcome of infection varies depending on complex interactions between virulence, tropism and pathogenicity of the infecting species and the immune response of the mammalian host (Mandell et al., 2004). Although a species can produce more than one of these clinical forms there is a predisposition for particular species to cause a certain disease phenotype (Table 1).

VL is a systemic disease mainly caused by two species: *L. donovani* and *L. infantum* (syn. *L. chagasi*; Assimina et al., 2008). From 0.5 million new cases that are estimated to occur annually in 65 countries, 90% of these are found in Bangladesh, Brazil, India, Nepal and Sudan, affecting both children and adults (WHO, 2010).

In this clinical form, the parasite has a tropism for internal organs, in particular the spleen, liver, bone marrow and lymph nodes. Most common clinical manifestations are undulating fever, weight loss, loss of appetite, spleno- and hepatomegaly. VL is considered the most severe form of disease, being potentially fatal if left untreated, with a mortality rate of almost 100% (Ezquerra, 2001; Assimina et al., 2008; Sharma and Singh, 2008).

Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL that usually develops 6 months to 1 or more years after apparent cure or in the absence of VL history. Its incidence is almost restricted to endemic areas of *L. donovani*, namely the Indian subcontinent and Sudan, where frequency of the condition is about 50-60% and 5-10%, respectively (Zijlstra et al., 2003; WHO, 2010; Ganguly et al., 2010).

CL, the most common form of human leishmaniasis, is a rarely fatal disease that is characterized by well-delimited skin lesions with elevated borders and it is estimated that its annual incidence is 1–1.5 million cases per year (Desjeux, 2004). The majority of cases are self-healing, although some lesions can persist and leave scars with variable size. A rare form of CL is diffuse cutaneous leishmaniasis (DCL) in which patients may have multiple mixed type skin lesions (non-ulcerative) in several parts of their body, within a short period of time since the appearance of the first lesions. It is difficult to treat, spontaneous cure is rare and can have devastating consequences to the individual (Turetz et al., 2002).

Table 1 Most clinically relevant *Leishmania* species, respective vectors, main geographic distribution and clinical features (Barral et al., 1991; Ziljstra et al., 2003; Reithinger et al., 2006; Alborzi et al., 2006; Akilov et al., 2007; Amato et al., 2008; Badirzadeh et al., 2013).

	Vector	Species	Main geographic distribution	Clinical Features
Old World	*Phlebotomus* spp.	*L. (L.) donovani*	East Africa and India subcontinent	VL*, CL**, PKDL*
		L.(L.) infantum	Southern Europe, North Africa, Central Asia and some parts of China	VL*, CL**, PKDL***
		L. (L.) major	India, North Africa, West Asia	CL*, MCL**
		L. (L.) tropica	Central Asia, Middle East, Southeast Asia and parts of North Africa	CL*, MCL**, VL***
		L. (L.) aethiopica	Etiopia, Kenia, Sudan	CL***,** DCL*
New World	*Lutzomyia* spp	*L. (L.) infantum (syn. L. chagasi)*	Latin America	VL*, CL*,MCL**
		L. (L.) mexicana	Mexico, Central America,Texas, Oklahoma (USA)	CL*, DCL**
		L. (L.) amazonensis	Amazon basin, Brazil	CL*, DCL**, MCL**, VL***
		L. (L.) venezuelensis	Venezuela	CL*
		L. (V.) braziliensis	South and Central America, Mexico	CL*, MCL*, DCL**
		L. (V.) panamensis	Panama, Costa Rica, Colombia	CL*, MCL*
		L. (V.) guyanensis	Northern Amazon basin, Guyana	CL*, MCL**
		L. (V.) peruviana	Peru and Argentina	CL*

VL, Visceral leishmaniasis; CL, Cutaneous leishmaniasis; DCL, Diffuse cutaneous leishmaniasis; MCL, Mucocutaneous leishmaniasis; PKDL, Post-kala-Azar dermal leishmaniasis. *common; ** rare; *** very rare.

MCL or espundia can be caused by various species. However, *L. braziliensis* is responsible for the majority of cases and, consequently, geographic distribution of this manifestation is almost restricted to South America. Severe MCL cases can lead to facial disfiguration, which is never self-healing, being difficult to manage and thus potentially fatal (Reithinger et al., 2006; Amato et al., 2008).

1.3.1. Leishmaniases in Portugal

The first autochthonous case of leishmaniasis in Portugal was described by Alvares (1910), referring to a 9-year-old female. A year later, the same author reported the occurrence of CanL in Lisbon, detecting an incidence of 2.66% upon examining 300 dogs. However, no infection was found, in the 58 cats screened (Alvares and Silva, 1911).

Since these reports, additional studies have been carried out in Portugal, describing the prevalence of VL, CanL and unraveling the bioecology and distribution of the vectors (Abranches et al., 1993; Cardoso, 2004a, 2004b; Marques, 2008; Branco et al., 2013; Sousa et al., 2011; Cortes et al., 2012).

L. infantum is the major responsible for the VL cases in Portugal, although few cases of CL caused by this species were also diagnosed in Portuguese adults and children. Concerning phlebotomine sand flies, besides of only *P. perniciosus* and *P. ariasi* are the confirmed vectors of *L. infantum*, *P. papatasi*, *P. sergenti* and *S. minuta* are known to also occur in the country (revised by Campino et al., 2006; Cortes et al., 2007; Campino and Maia, 2010).

VL cases in the Mediterranean basin usually affect pediatric patients less than 4 years old and human immunodeficiency virus (HIV) co-infected individuals (Kafetzis, 2003; WHO, 2010). In fact, in Southern Europe, 70% of VL cases in adults are related to HIV-positive individuals, especially among intravenous drug users. Most probably needle-sharing has inadvertently created an alternative cycle of transmission, putting this population at risk for contracting the disease (Cruz et al., 2006). In Portugal, the Lisbon Metropolitan Area is considered to be a focal point of the disease, where most cases in humans have been reported, especially due to HIV co-infection, as previously mentioned (Campino and Maia, 2010).

Between 2000 and 2006, 152 new cases of VL were diagnosed in the Unidade de Leishmanioses, IHMT (26% in immunocompromised individuals), but in the same

period, only 86 cases of VL were notified to Direcção Geral de Saúde, 74 of which occurring in the Lisbon Metropolitan Area (Cortes, 2008). However, between 1999 and 2009, only 145 cases of VL were notified through the mandatory disease declaration system (Serrada, 2010). Thus, these data suggest that the number of actual cases may be underestimated.

CanL is endemic in the Mediterranean basin being considered a serious public health problem mainly due to subclinical infections that delay the diagnosis favoring the maintenance and transmission of disease (Solano-Galego et al., 2011). The situation of CanL in Portugal is more disturbing than of human leishmaniasis. Three regions, namely, Alto Douro, Lisbon and Algarve, are known endemic foci of CanL. In some localities such as Alijó, the prevalence of infection can reach up to 20% (Campino et al., 2006). In a study performed by Cortes et al. (2012), the dog's age, fur size, district and living outdoors were the risk factors identified for CanL in Portugal. Moreover, in the same study, higher seroprevalence was found in districts from the interior of Portugal, namely Castelo Branco, Beja and Portalegre, in comparison to coastal regions (Cortes et al., 2012).

Considering the biological cycle of *Leishmania* parasites, an increase in CanL cases should not be take too lightly, because it can foretell an increase in human visceral leishmaniasis cases.

1.4. Current available and under development drugs for leishmaniases treatment

The control of this vector-borne disease relies mostly in chemotherapy till date. However, current available drugs for human leishmaniasis treatment exhibits several disadvantages such as high costs, toxicity, long-term periods of parental administration for some drugs, variation in intrinsic sensitivity between *Leishmania* species and development of drug resistances (Croft and Coombs, 2003; Singh et al., 2012).

Table 2 First and second-line drugs used against leishmaniases and their possible or established mode of action (Saha et al., 1986; Vercesi and Docampo, 1992; Maarouf et al., 1995; Balaña-Fouce et al., 1998; Sereno et al., 2001; Sudhandiran and Shaha, 2003; Bray et al., 2003; Paris et al., 2004; Verma and Dey, 2004; Chawla et al., 2011; Fernández et al., 2011; Carvalho et al., 2011).

Drug	**Mode of Action**
Pentavalent antimonials	Conversion to its toxic trivalent (Sb^{III}) form within macrophage inhibits trypanothione reductase and exposes parasite to oxidative stress. DNA fragmentation, PS externalization, ROS generation, and increase of Ca^{2+} levels were observed.
Miltefosine	Unclear. Induces apoptosis-like cell death – based on observed phenomena like cell shrinkage, DNA condensation and fragmentation and PS externalization.
Pentamidine	Unclear. Disintegration of kinetoplast and mitochondria and collapse of the mitochondrial membrane potential were observed.
Amphotericin B	High affinity to ergosterol in *Leishmania* membrane, forming channels-like pores leading to permeability of the membrane and causing parasite death.
Paromomycin	Inhibits protein synthesis by targeting proteins involved in translation, particularly ribosomal proteins.
Sitamaquine	Acts against the respiratory chain – based on observed phenomena like induced depolarization of membrane potential, increased ROS production, increased Ca^{2+} levels and PS externalization.

PS – Phosphatidylserine; ROS – Reactive oxygen species

For more than 60 years, pentavalent antimonials compounds (Sb^{v}), sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), remained as first line drugs of choice against all forms of leishmaniasis, although their action mechanisms are still unclear (Singh et al., 2012; Table 2). Intrinsic variation between

Leishmania species have been pointed out as the reason for variation in clinical response to Sb^v treatment. As revised by Croft et al. (2006), *L. braziliensis* and *L. donovani* seem to present higher sensivity to Sb^v than *L. tropica*, *L. major* and *L. mexicana*.

First reports of pentavalent antimonials resistance came from Bihar, India in 1980's when patients were not responding to the treatment. Nowadays, half of the global cases of VL occur in North Bihar and India, which is considered as an endemic area displaying high drug resistance, mainly due to inadequate dosage or incomplete treatment duration (Sundar, 2001; Tiuman et al., 2011). Nowadays, second-line drugs include miltefosine, amphotericin B (AmB) and pentamidine.

In 2014, miltefosine (Impavido®) was approved by Food and Drug Administration (FDA) to treat the three main forms of leishmaniasis, but especially VL caused by *L. donovani* (FDA, 2014). Regardless of its dual effectiveness, it has the disadvantage of requiring a long period of administration with some side effects such as vomiting and diarrhea with occasional hepatic and renal toxicity, which can lead to abandonment of treatment and non-compliance by patients. Moreover, because of its teratogenicity, it is not suitable for pregnant women (Singh et al., 2012; Ponte-Sucre et al., 2013).

Miltefosine also showed to be well tolerated and therapeutically efficient against CanL, in a clinical trial with *L. infantum* infected dogs from the Mediterranean basin (Woerly et al., 2009). Still, the increase of its off-label use to treat CanL, in Europe, may lead to *L. infantum* parasite's resistance.

AmB is an antifungal drug with high affinity to ergosterol, the main sterol in fungal and tripanosomatids cell membrane, that is used in endemic areas with parasite resistances reported (Singh et al., 2012). In Europe, AmB is widely used as drug of choice in the treatment of visceral cases, especially in pedriatic and HIV co-infected individuals (Maia et al., 2009). Due to side effects such as fever and renal complications of AmB, liposomal formulations of AmB have been developed and proved to be highly efficient. However, its high costs limit their application (Singh et al., 2012).

Phase II clinical trials in India and Africa suggested that the oral drug sitamaquine was effective and well tolerated for treatment of VL, despite some mild adverse effects reported, such as vomiting, dyspepsia, cyanosis, nephritic syndrome, glomerulonephritis,

abdominal pain, headache and severe renal dysfunction (Jha et al., 2005; Wasunna et al., 2005).

Paromomycin is an aminoglycoside that may have ribosomes of *Leishmania* as its primary cellular target (Maarouf et al., 1995). A combination of gentamicin and paromomycin for topical use against CL was evaluated in phase III clinical trials, and the use of both paromomycin with or without gentamicin was efficient (Ben Salah et al., 2013).

In addition to the drugs here described, the number of patents for antileishmanial compounds has dramatically increased since 1980's up to now. However, because of the shortcomings listed above, there is still an urgent need of novel, less toxic, more affordable and effective anti-*Leishmania* drugs with short-term administration periods that can be used alone or in combination with other therapies (Monzote, 2011).

2. Natural Products in Drug Discovery

The term natural product (NP) refers usually to secondary metabolites produced by living organisms, which are generally derived biosynthetically from primary metabolites (lipids, aminoacids) and are not essential to growth, development and reproduction of the living organism, but they may influence ecological interactions between these and the environment where they are inserted (Buchanan et al., 2000).

NP have extensively been studied in the past decades for their biological activities for medicinal, recreative and nutritional applications (Ramawat and Mérillon, 2008). Plants have always been a rich source of NPs are still being used in traditional medicine for their medicinal properties mainly in developing countries from Africa, Latin America and Asia, and ethnobotanic studies have been carried to support the discovery and development of new drugs (Valentin et al., 2000; Sheng-Ji, 2001).

Interestingly, the antiparasitic potential of plants used in traditional medicine has been investigated. The most successful case is *Artemisia annua*, a plant that has been used for more than two thousand years in China's traditional medicine as a remedy for fever. It has been shown that its major active compound, artemisinin, can be used efficiently against malaria, avoiding many deaths since mid-70s until now (Woerdenbag et al., 1990).

Apart from the diversity of terrestrial plants, water covers 70% of our planet and in some ecosystems the biodiversity is higher than that in rain forests. Therefore the potential of marine organisms, such as invertebrates and algae, has been recognized as sources of novel drug leads (Haefner, 2003).

In the last decade, over than 5,000 structures of marine natural products have been described, many with potential to be applied in several fields (Sarker et al., 2006). Still, more than 1,000 molecules have already been developed, some of which are currently under clinical evaluation for the treatment of cancer (Newman and Cragg, 2004), Alzheimer's disease (Won Kim et al., 2007) and other ailments.

Marine natural products have been obtained from cyanobacteria, actinomycetes, fungi, algae, sponges, corals and ascidians. Between 2008 and 2010, from the 133 antiparasitic small molecules analyzed with an inhibitory concentration of 50% (IC_{50}) below 30 µM, 87 apparently derived from marine sponges (Watts et al., 2010).

According to Mayer et al. (2011), between 2007 and 2011, sixteen studies of marine products contributed to novel findings in antiprotozoal pharmacology. Six studies focused on *Leishmania* spp. with an IC_{50} between 0.093 and 12 µg mL^{-1} (Mayer et al., 2011; 2013).

Although many studies have been focusing in screening marine organisms and promising results have been published, only few antiprotozoal compounds have been identified in the last decades (Watts et al., 2010).

2.1. Algae as source of antileishmanial compounds

There are about 30,000 species of algae all over the world and approximately 13,000 are macroalgae. These are usually distinguished morphologically on the basis of the thallus colour: red (phylum *Rhodophyta*), green (phylum *Chlorophyta*) and brown (phylum *Ochrophyta*) algae (Bhakuni and Rawat, 2005; Hayes, 2012).

Macroalgae are exposed to several abiotic and biotic stresses and so their bioactive compounds have evolved as chemical weapons in response to adversities. Therefore they may show multiple functions and biological activities (Hayes, 2012).

Several studies have demonstrated the potential of seaweeds bioactive metabolites with anticancer (Hussain et al., 2012), antioxidant (Vadlapudi, 2012), anti-inflamatory

(Lee et al., 2013), antifungal (Cosoveanu et al., 2010), antibacterial (Tuney et al., 2006), antiprotozoal (Allmendinger et al., 2010) and antifouling (Manilal et al., 2010) activities.

Recently, investigators have screened many algae from all over the world against different species and stages of *Leishmania* (Table 3).

In these studies, the same algal species from different places presented diverse antileishmanial activities, possibly due to different metabolites that are produced in response to different environment conditions (Orhan et al., 2006; Spavieri et al., 2010b).

The majority of the reports that studied antileishmanial activity, characterized the activity of crude extracts and very few have been tested in their pure form, namely against *L. amazonensis* (Dos Santos et al., 2010; Da Silva Machado et al., 2011; Dos Santos et al., 2011; Soares et al., 2012). The identified compounds are the sesquiterpene elatol, isolated from the red alga *Laurencia dendroidea* (Dos Santos et al., 2010), the sesquiterpene obtusol also from *L. dendroidea* (Da Silva Machado et al., 2011), the 4-acetoxy-dolastane diterpene obtained from the brown alga *Canistrocarpus cervicornis* (Dos Santos et al., 2011) and the dolabellene diterpene dolabelladienetriol, purified from the brown alga *Dictyota pfaffii* (Soares et al., 2012). All presented remarkable IC_{50} values and curiously, dolabelladienetriol, besides inhibiting the growth of *L. amazonensis* promastigotes and intracellular amastigotes, it also reduced the parasite growth during HIV co-infection (Soares et al., 2012). Also, in these studies, different parasite forms demonstrated different susceptibilities to the same algal extract, highlighting the importance of a suitable *in vitro* model.

Table 3 Marine algae species with antileishmanial activity and IC_{50} below 20 μg mL^{-1}. (Adapted from Vizetto-Duarte et al., *in press*).

Macroalgae species	***Leishmania* spp.**	**Parasite stage**
Codium fragile ssp. Tomentosoides	*L. donovani*	AA
Ulva intestinalis	*L. donovani*	AA
Ulva lactuca	*L. donovani*	AA
Bifurcaria bifurcata	*L. donovani*	AA
Cystoseira baccata	*L. donovani*	AA
Cystoseira tamariscifolia	*L. donovani*	AA
Halidrys siliquosa	*L. donovani*	AA
Dictyopteris polypodioides	*L. donovani*	AA
Dictyota dichotoma	*L. donovani*	AA
Turbinaria turbinata	*L. mexicana*	P
Laurencia microcladia	*L. mexicana*	P
Ceramium rubrum	*L. donovani*	AA
Halopitys incurvus	*L. donovani*	AA
Dilsea carnosa	*L. donovani*	AA
Scinaia hatei	*L. major*	P
Laurencia pinnatifida	*L. major*	P
Bostrychia tennela	*L. mexicana*	P

AA – axenic amastigotes; P – promastigotes

2.1.1. Brown macroalgae belonging to the *Cystoseira* genus

The family *Cystoseiraceae* (Order *Fucales*) have a wide distribution in the Northern Hemisphere. Nevertheless, from 292 of brown algae species known belonging to the *Cystoseira* genus, 80% occur along the Mediterranean and Atlantic coasts (Garreta et al., 2001; Guiry and Guiry, 2014).

In Portugal, the most common species are *C. baccata*, *C. humilis*, *C. nodicaulis*, *C. tamariscifolia* and *C. usneoides* (Garreta et al., 2001).

Since 1984, *Cystoseira* spp. have been chemically studied extensively. Its chemical profile characterization revealed mainly diterpenoids (Ayyad et al., 2003), meroditerpenoids (De Los Reyes et al., 2013), tetraprenyltoluquinol derivatives (Fisch et al., 2003), fatty acids, sterols (Andrade et al., 2012) and terpenes (Kamenarska et al., 2002).

Considering its chemical constitution, *Cystoseira* species have been investigated for their pharmaceutical potential and among those studies extracts from these algae were shown to possess antibacterial (Bennamara et al., 1999; Süzgeç-Selçuk et al., 2010; Spavieri et al., 2010a), antifungal (Bennamara et al., 1999), antiprotozoal (Spavieri et al., 2010a; Süzgeç-Selçuk et al., 2010), antioxidant (Fisch et al., 2003; Mhadhebi et al., 2011), cytotoxic (Ayyad et al., 2003; Spavieri et al., 2010b), antiviral (Urones et al., 1992), anti-tumoural (Urones et al., 1992) and anti-inflammatory (Mhadhebi et al., 2011; De Los Reyes et al., 2013) activities.

Nevertheless, few papers that evaluate the antileishmanial activity of novel *Cystoseira* species have been published. For example, *C. baccata*, *C. tamariscifolia, C. barbata* and *C. crinita* extracts have been shown to be active against *L. donovani* axenic amastigotes (Spavieri et al., 2010b; Süzgeç-Selçuk et al., 2010). Only two studies have evaluated the potential of macroalgae from the *Cystoseira* genus against *L. infantum* (Bruno de Sousa et al., 2012; Ainane et al., 2014).

The published results together with the availability and biodiversity of the *Cystoseira* species in the Iberian Peninsula suggest that this genus is a potential source of antileishmanial compounds.

2.2. The pharmacological potential of halophytes

Halophytes are a group of plants that can tolerate high salinity levels, occurring in a variety of environments such as sand dunes, rocky coasts, saline depressions or inland deserts but also in locations with sea influence, such as coastal regions, salt marshes and mudflats (Ksouri et al., 2012a).

There are about 2,600 species of halophytes, 700 of which occurring in the Mediterranean flora (Beligno and Sardo, 2008). Besides being a relative small group of plants, the research on their biological metabolites remains quiet unexplored. Because of the extreme conditions that they encounter in their habitats (salinity, drought, UV light),

halophytic plants have developed several biochemical defenses by biosynthesizing a variety of bioactive compounds such as polyphenols (Ksouri, 2012b) saponins, flavonoids and sterols (Ksouri et al., 2013).

Also in response to biotic and abiotic stress, halophytes may accumulate high levels of ROS, which can lead to cell damage. However, their potent antioxidant system is able to overcome and quench toxic ROS, suggesting that this may be an environmental adaptation (Ksouri et al., 2012b; Mohammed et al., 2013). Thus, several studies have reported the antioxidant potential of halophytes.

Recently, five halophyte species from southern Portugal demonstrated powerful antioxidant, anti-inflammatory and anti-tumoral activities (Rodrigues et al., 2014). Furthermore, *Arthrocnemum macrostachyum* and *Carpobrotus edulis* have been described as powerful sources of compounds against degenerative and neurological disorders such as Alzeihmer's disease (Custódio et al., 2012).

Other biological activities have been reported such as antibacterial (Ibtissem et al., 2012), antifungal (Samiullah and Bano, 2011), antifouling (Kong et al., 2014), antiobesity (Kalai et al., 2013) and antidiabetic (Chikhi et al., 2014).

Besides some halophytes species are described in the literature as used in folk medicine to treat parasitic diseases, no published studies exploring the activity of halophyte extracts or derived compouds towards *L. infantum* parasites were found (Farooq et al., 2008). However, only a panel presentation studied the antileishmanial activity of *Carpobrotus edulis*, *Juncus acutus* and *Convolvulus soldanella* (Bruno de Sousa et al., 2013).

In conclusion, reports concerning the antiparasitic activity, more specifically antileishmanial activity are scarce. Nevertheless, their pharmacological potential against *Leishmania* spp. should not be ignored, having in mind their biodiversity, availability and the wide range of demonstrated bioactivities.

3. *In vitro* screening of natural products with antileishmanial activity

Plants and marine organisms possess several compounds in its constitution. Thus, the isolation of NP can be time-consuming, laborious and also may depend on the separation techniques and solvents used (Sticher, 2008).

Diverse extraction methods can be applied, according to the compounds of interest and the aim of its isolation (Cannel, 1998). A critical first step in NP isolation is the

preparation of extracts, complex mixtures of compounds, using different organic solvents (e.g., hexane, acetone, dichloromethane, methanol, ethanol; Tempone et al., 2011).

Different solvents will extract different compounds, according to the solvents' polarity and the compounds solubility, which may affect its bioactivity. For example, the use of 80% acetone (ACE) is suggested to extract a higher concentration of phenolic compounds instead of 80% methanol (MeOH) and ethanol extraction, which reflects in the antioxidant activity of the extracts (Zhao et al., 2006; Taha et al., 2011). Moreover, more polar solvents will extract hydrophilic compounds and, in contrast, lypophilic compounds can be extracted using, for example, dichloromethane (Cos et al., 2006).

Thus, the selection of the extraction method is of major importance to an efficient *in vitro* screening of antileishmanial activity. Despite the simple procedures of conventional extraction methods, such as percolation, maceration and Soxhlet-based extractions, these may present several drawbacks like the need for large amounts of solvents, being time-consuming and leading to differences in extraction efficiency (Sticher, 2008). Also, it is essential to avoid the lost or degradation of volatile and labile compounds, during the preparation of extracts that can be potentially active (Cos et al., 2006; Sticher, 2008).

The primary screening approach to identify the potential of extracts as source of antileishmanial compounds include two essential techniques: determination of the antileishmanial activity and cytotoxicity. Usually, the efficiency of bioactivity of an extract is evaluated by the IC_{50}. Therefore, an interesting extract will exhibit a high activity against the parasite and low toxicity against cells, based on the IC_{50} values, being considered selective against *Leishmania* parasites.

Moreover, as the compound(s) responsible for the activity observed against *Leishmania* parasites may differ from the one responsible for the cytotoxicity, some authors only evaluate the cytotoxicity of the selected fraction(s) or compound (s), after bio-guided fractionation studies (De Toledo et al., 2014).

Once the extract demonstrates to possess active and selective compounds against *Leishmania* parasites, it can be fractioned and the activity of each fraction assessed until a pure fraction, containing the active compound(s), is obtained. Extracts and fractions must be tested at a maximum concentration of 300 µg mL^{-1} and isolated compounds at maximum of 100 µg mL^{-1} in order to avoid false selection of poorly active compounds

(Tempone et al., 2011). However, it has been proposed that concentrations higher than 150 µg mL^{-1} usually cause an increase in false positive (Cos et al., 2006).

The main difficulty of researching novel antileishmanial compounds still lies on the lack of sensitive, rapid and standardized screening methods. Nowadays, different techniques are being used, focusing on axenic promastigotes, amastigotes and intracellular amastigotes (Spavieri et al., 2010a; Jain et al., 2012; Feily et al., 2012).

3.1. Parasite and cellular models for *in vitro* assays

In vitro assays focusing on the promastigote stage are widely used for preliminary screens mainly due to its simple maintenance in laboratory. Moreover, the screening method usually requires little amounts of extracts and fractions and do not require sophisticated equipaments, allowing the test of several samples simultaneously (Tempone et al., 2011).

However, the use of the promastigotes form in screening assays has been controversial since it is the extracellular form, mainly found in the phlebotomine vector, requires different environmental conditions than those for amastigotes. Consequently, is considered that it may not be a suitable *in vitro* model (Gupta and Nishi, 2011). Supporting this idea, a high percentage of compounds that were active against promastigotes were found to be inactive against intracellular amastigotes and vice versa, resulting in false positive and false negative results (Siqueira-Neto et al., 2010; De Muylder et al., 2011).

Axenic amastigotes are also easy to maintain in cultured suspensions in laboratory. Because of its similarity to the relevant stage of the parasite, these have been pointed out as promising *in vitro* models for drug screening. However, the activity against axenic amastigotes can be underestimated since it does not consider either the extreme intracellular conditions inside the macrophage or the compound penetration into the host cell (Gupta and Nishi, 2011; Tempone et al., 2011; Bringmann et al., 2013). For instance, naloxonazine was found to be active against intracellular amastigotes but inactive against both axenic forms of *L. donovani*, suggesting that its activity may be dependent of the presence of the host cells (De Muylder et al., 2011). In addition, compounds active against axenic amastigotes can be inactive against intracellular forms, due to the inability of these compounds to penetrate the macrophage membrane or to withstand the acidic

environment inside the host cell (De Muylder et al., 2011). Thus, the presence of the host cell could be important to precise the drug-mediated toxicity towards the intracellular parasites (Sereno et al., 2007).

Not surprisingly, the use of both axenic forms have been questioned mainly due to relevant differences in cellular, physiological, biochemical and molecular levels when compared to intracellular forms (Jain et al., 2012).

In contrast, intracellular amastigotes are obtained by *in vitro* infection of mammalian cell lines with the parasite. Concerning its mammalian host, *Leishmania* infect mononuclear phagocytic cells, mainly differentiated macrophages. These descend from monocytes recruited from blood, being a crucial component of the innate immune response (Vannier-Santos, 2002; Daigneault et al., 2010). Thus, various cellular types are used as host cells such as murine peritoneal macrophages or human-monocyte transformed macrophages such as L6, THP-1 and J774, available as continuous cultures. As the latter are monocytic cellular lines, they require differentiation into macrophages and loss of proliferation, to assure that are no confounding effects when activity of the compound is assessed (Ioset et al., 2009; Gupta and Nishi, 2011).

To achieve differentiation stimuli such as phorbol-12-myristate-13-acetate (PMA) have been used. PMA is a phorbol ester, activator of protein kinase C that is widely used to induce cell differentiation, hence it increases adherence and expression of surface markers and promotes the loss of proliferation, associated with macrophage differentiation (Schewnde et al., 1996; Daigneault et al., 2010).

Recently, the comparison between the antileishmanial activities of four standard drugs against intracellular amastigotes of *L. donovani* highlighted the influence of the type of host cell used and *in vitro* activity observed (Seifert et al., 2010).

3.2. Methods applied to antileishmanial drug discovery

Cell viability based assays, such as resazurin or 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), are the most commonly used assays in a laboratory setting to evaluate antileishmanial activity in parasite axenic forms or cytotoxicity against host cells. This is mainly due to their low costs, accuracy and the ease with which dose response curves can be obtained (Tempone et al., 2011). The principle of these assays relies on the metabolization of the active compounds (resazurin, MTT) by viable cells,

which enables the fluorimetric and/or colorimetric quantification of the percentage of viable cells.

Classical methodologies for the evaluation of antileishmanial activity of compounds in intracellular amastigotes include direct counting assays after stained smears and the determination of the inhibitory concentration values, based on the percentage of infected cells or number of amastigotes per macrophages counted (Sereno et al., 2007). This method remains the standard procedure. However, it has several drawbacks such as being time-consuming, laborious and may provide subjective operator-dependent microscopic counts that can lead to inaccurate evaluations. Moreover, these techniques are usually used to assess the activity of compounds which demonstrated previously to be active against axenic forms (Fumarola et al., 2004; Tempone et al., 2011).

For years, several attempts were made to develop an ideal method for screening against the clinical relevant stage, including labelling parasites with monoclonal antibodies (Guinet et al., 2000) and with radioactive uracil nucleotides (Berman and Gallalee, 1985). However, the significant short-comings of these techniques, mainly high costs and the use of radioactive nucleotides may represent a potential risk for workers, which have led to their scientific oblivion (Sereno et al., 2007).

More recently, Jain et al. (2012) described a new fluorimetric method based in parasite rescue of *L. donovani* intracellular amastigotes. Briefly, after infection of differentiated THP-1 cells and treatment with reference drugs, a critical step is the lysis of the macrophages with sodium dodecyl sulfate (SDS) and mechanic agitation of the plate, enabling the release of intracellular amastigotes. After this step, plates are incubated for amastigotes to return to the promastigote form and its viability is evaluated with the commercial resazurin-based kit, Alamar Blue® (Jain et al., 2012).

Resazurin is a blue dye, used as a cell viability indicator which is reduced by viable cells to the fluorescent dye, resorufin (O'Brien et al., 2000). This dye has gain the interest due to its appealing advantages. Resazurin is relatively non-toxic to cells, less expensive, allows the choice of the detection method (colorimetric/fluorimetric) and does not require a centrifugation step when working with non-adherent cells, which reduces manipulation errors (Tempone et al., 2011). Moreover, it has already been used as a viability indicator on antileishmanial drug screening assays towards axenic amastigotes as well as promastigotes (Spavieri et al., 2010a; Kulshrestha et al., 2013).

Over the last few years, the reporter gene technology has emerged as a valuable, sensitive and faster tool for quantifying the growth of intracellular forms. This technology is based on parasites transfected with a gene that has a measurable phenotype. Diverse reporter genes are mentioned in the literature including green or red fluorescent protein (Rocha et al., 2013), β-galactosidase (Okuno et al., 2003), β-lactamase (Mandal et al., 2009) and firefly luciferase (Lang et al., 2005). Nevertheless, these assays are far from ideal. Not only they require sophisticated equipment, but also these genes may affect the parasite's biology (Tempone et al., 2011).

Regarding all difficulties inherent to drug screening against intracellular amastigotes, a rapid and sensitive screening method focusing on this parasite form would improve the efficacy of research of novel drugs against leishmaniasis. Although this hypothesis has been considered to be unreachable, the present work is a novel contribution towards this difficult goal.

4. Thesis justification

Intracellular amastigotes are pointed out as the ideal *in vitro* model for assessing the effect of antileishmanial drugs or compounds. These cells are obtained by infection of mammalian cell lines or peritoneal macrophages with both axenic promastigotes and amastigotes (Maia et al., 2007; Veermesch et al., 2009). Despite being labor-intensive, time-consuming, subjective, and dependent on the establishment of an amastigote-macrophage system, the direct counting method still remains the gold standard methodology to evaluate the antileishmanial activity of natural products towards the clinically relevant stage of *Leishmania* parasites (Yazdanparast et al., 2014). The optimization process of this method depends on some critical steps, such as differentiation of the monocytic cellular line into adherent macrophages, infection with *Leishmania* promastigotes, treatment with the extracts or compounds and Giemsa staining of the slides. Because of its drawbacks, its use is usually limited to test compounds with verified antileishmanial activity in axenic promastigotes and/or amastigotes. However, as the use of these forms can result in missing potential hits, the attempt to use novel methodologies focusing on this parasite form is clearly needed.

In this context, the parasite rescue method described by Jain et al. (2012) offers an interesting alternative to testing large numbers of extracts or compounds in a rapid, affordable, and sensitive manner, and may give more appropriate results. Nevertheless,

this methodology may require extensive optimization, in particular when experimental protocols for screening bioactive compounds need to be adapted to a novel target *Leishmania* species, as is the case for *L. infantum*. Still, the implementation of this method in laboratory routine is an undoubtedly a useful tool in the search for novel bioactive compounds.

Considering that a majority of published studies have shown antileishmanial activity of marine extracts and having in mind the potential of marine organisms from the Iberian coast as sources of novel compounds with cytotoxicity to *L. infantum* has yet to be fully explored, a thesis on amastigotes-based assay optimization and screening for bioactive extracts against this parasite, clinically revelant to Portuguese human and canine populations, is needed.

5. Objectives

The main goal of this work is to contribute to the study of the potential of marine organisms and land plants living in habitats with marine influence as sources of compounds with antileishmanial activity. This will be attempted by optimizing screening methods, in particular those involving intracellular amastigotes. To accomplish this goal, several specific objectives were defined, namely: 1) optimization of the mammalian cell infection model; 2) optimization of two screening methods focusing on intracellular amastigotes of *L. infantum*, namely the direct counting upon Giemsa staining and and adaptation of the parasite rescue assay; 3) preparation of the macroalgae and halophyte extracts; 4) determination of the activity of macroalgae and halophyte extracts towards *L. infantum* promastigotes; 5) determination of the extracts cytotoxicity towards an *in vitro* mammalian model and selectivity indexes of the extracts; and 6) evaluation of the activity of the extracts in the macrophage-amastigote system.

Chapter II – Material and Methods

This work was developed in the Marbiotech group of the Centre of Marine Sciences located at the Universidade do Algarve in collaboration with the Unidade de Leishmanioses of the Instituto de Higiene e Medicina Tropical (IHMT).

The following procedures on this chapter were taken under aseptic conditions, in a laminar flow cabinet fitted with HEPA filters.

1. Amastigote-macrophage model

1.1. Parasites and cell cultures

Human acute monocytic leukemia cell line (THP-1; ATCC® TIB-202™) and the human visceral *Leishmania infantum* strain MHOM/PT/88/IMT-151 were used in this study. Both cultures were kindly provided by the Unidade de Leishmanioses, Unidade de Parasitologia Médica, IHMT.

Both cell line and parasite cultures were maintained in RPMI medium with L-glutamine (Lonza) supplemented with 10% heat inactivated fetal bovine serum (FBS; Biochrom) and 1% penicillin (50 U mL^{-1})/streptomicin (50 µg mL^{-1}; Pen/Strep; Lonza) at 36ºC ± 1ºC in 5% CO_2 and 24 ± 1ºC, respectively. THP-1 flasks were incubated horizontally with ventilated cover and *L. infantum* flasks were incubated at an angle, to increase oxygenation. The medium was changed every 3 days.

For infection assays, THP-1 and *L. infantum* promastigotes were maintained in RPMI without medium change for 4 and 5 days, respectively. Maintenance of cell and parasite lines is crucial to guarantee the availability of large quantities of cells but also to ensure that the *L. infantum* parasites were at the desired development phase for each assay. All infection assays were incubated at 36ºC ± 1ºC in 5% CO_2.

To avoid loss of virulence by the parasites as described elsewhere (Moreira et al., 2012), experiments were carried out with parasites with less than 10 passages, after the first isolation from the murine model, whenever possible.

1.2. Seeding and differentiation of mammalian cells

As discussed in I-3.2, since THP-1 is a monocytic cellular line, differentiation of these cells into adherent macrophages is required for infection assays and has been addressed by treating cells with PMA (Daigneault et al., 2010).

To each assay, exponentially growing THP-1 cells (2,5 x 10^5 cells mL^{-1}) were seeded in sterile 16-chamber, glass, microscopic culture slides (Millicell® EZ SLIDE). PMA (Sigma) was added to cell suspension at a final concentration of 25 ng mL^{-1} (Jain et al., 2012). To each well, 200 µL of the diluted cell suspension were dispensed and the chamber was incubated for 24 h, to allow the differentiation of THP-1 cells into adherent macrophages (De Muylder et al., 2011; Jain et al., 2012).

1.3. *In vitro* infection of mammalian cells with *L. infantum* promastigotes

1.3.1. Growth curve and relative percentage of metacyclic forms

As mentioned before in I-1.1.1., metacyclic promastigotes are the infective forms of *Leishmania* spp. Therefore, for the determination of the day on which promastigotes should be added to the seeded adherent macrophages for infection, the growth dynamic and percentage of metacyclic forms in culture were assessed.

L. infantum promastigotes cultures were maintained as described in II-1.1., and the parasites' concentration was adjusted to 1,0 × 10^6 promastigotes mL^{-1}. In a 12-well plate, 1 mL of culture was dispensed per well. To avoid cross-contamination, it was left one well between replicates. Cultures were followed for 9 days and the concentration of the parasite was determined by counting the number of viable promastigotes, in triplicate, using a Neubauer's hemacytometer (Albuquerque, 2013).

Metacyclic promastigotes were counted simultaneously, based on morphological parameters such as its slender body, elongated flagellum and high motility (Bates and Tetley, 1993), and its percentage was determined with reference to the total number of viable promastigotes counted.

At day 3 and 5 of the growth curve, corresponding to logarithmic and stationary phases, respectively, *L. infantum* promastigotes were smeared on slides, stained by Giemsa (10% *v/v*; 10 min), observed in a light microscope (Motic BA310) and digital images were acquired for morphological analysis.

1.3.2. Determination of the optimal parasite:cell ratio

For the optimization of infection conditions, diluted suspensions of *L. infantum* promastigotes at the infective metacyclic stage were prepared with RPMI at 2% FBS, at 5 different parasite:cell ratios, namely 1.25:1, 2.5:1, 5:1, 10:1 and 20:1.

THP-1 cells were seeded and differentiated in chamber slides, as described in II-1.2. After differentiation, adherent macrophages were washed with 200 µL of serum-free RPMI, to remove the excess of PMA and 200 µL of the each diluted parasite suspension were added. The chamber slide was then incubated for 24 h. Non-infected control cells were included as negative control (De Muylder et al., 2011; Jain et al., 2012).

After 24 h, wells were washed five times with 200 µL of serum-free RPMI medium, to ensure the removal of non-internalized promastigotes. The plastic chamber was detached from the slide, which was left at the laminar flow cabinet for drying. When dried, the slide was immersed in MeOH for 30 s, washed with distilled water (H_2Od) and left to dry, before being stained by Giemsa (10% *v/v* in H_2Od, 10 min; Sigma).

The slides were observed by light microscopy under oil at a x1000 magnification and the percentage of infected cells (i.e. number of infected macrophages per 100 macrophages counted) and the intensity of infection (i.e. number of amastigotes per infected cells) were determined (Maia et al., 2007).

1.3.3. Determination of the optimal infection period

Since some reports are available in the literature that refer that lower times of incubation are sufficient in order to obtain good infection rates (Dasgupta et al., 2003; Maia et al., 2007; Jain et al., 2012), three different times were tested: 4, 16 and 24 h. Therefore, THP-1 cells were seeded and differentiated in chamber slides as described in II-1.2. and infected as referred to in II-1.3.2, using the optimal parasite:cell ratio. The chamber slides were incubated for 4, 16 and 24 h. Afterwards, slides were dried, stained with Giemsa and the percentage of infected cells and the intensity of infection were determined as described in II-1.3.2.

2. Intracellular susceptibility assays

One of the aims of this work was the optimization of two methods focusing on intracellular amastigotes: the direct counting method after Giemsa staining and an adaptation of the parasite rescue assay described by Jain et al. (2012).

2.1. Direct counting method

After establishment of the amastigote-macrophage model, to ensure that the method was prepared to test the effect of extracts, the effect of the standard drug AmB was evaluated, as positive control.

2.1.1. Evaluation of the effect of the standard drug, AmB

The THP-1 cells were seeded and differentiated as described in II-1.2. and infected for 24h at a ratio of 10:1 as described in II-1.3.2 (Figure 4). Six diluted suspensions of AmB were prepared, ranging from 4 to 0.0625 µg mL^{-1}, obtained from serial dilutions (1:2) with RPMI medium at 2% FBS. Non-treated cells were included as negative control. After 5 washes, 200 µL of each suspension was added to each well and the chamber was incubated for 48 h. When the treatment period ended, the chamber was washed three times with serum-free RPMI, detached and stained with Giemsa.

The percentage of infected cells and the intensity of infection were determined as described in II-1.3.2., and the inhibitory concentration (IC_{50} value, µg mL^{-1}) was calculated.

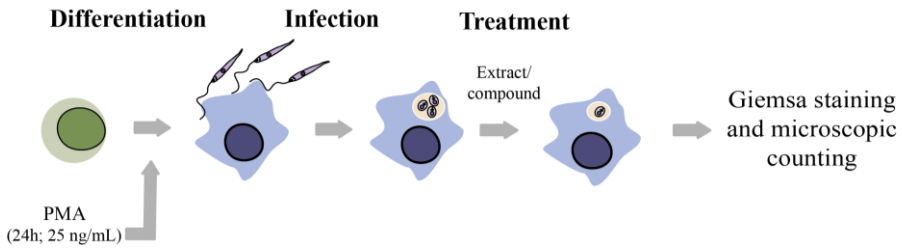

Figure 4 Schematic representation of the steps involved in the direct counting method (Original from Oliveira, M.).

2.2. Parasite rescue assay

Because of the drawbacks of the direct counting method, it was proposed to adapt the parasite rescue method developed by Jain et al. (2012), discussed earlier in I-3.2., by adding the resazurin immediately after lysis of infected cells and measure the viability of released amastigotes, eliminating the 48 h step for the transformation of the parasites.

2.2.1. Optimization of the controlled cell lysis

The procedure of this methodology is similar to the direct counting method till the treatment of the infected cells. Afterwards, a critical step is the controlled cell lysis using SDS solution and mechanic agitation of the plate (Figure 5).

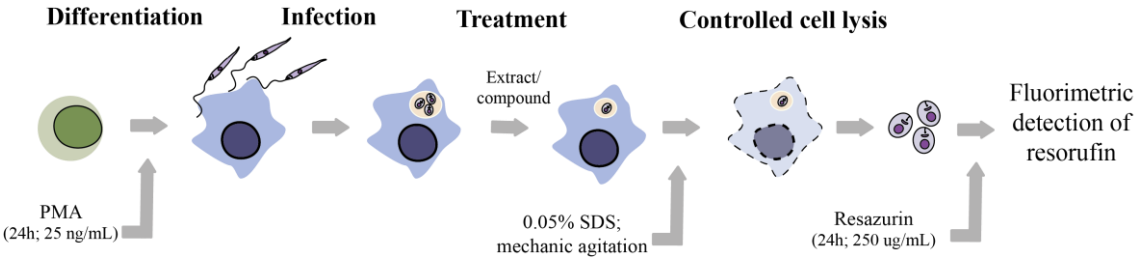

Figure 5 Schematic representation of the purposed adaptation of the parasite rescue assay (Original from Oliveira, M.).

To assess the optimal time for agitation of the plate after adding the SDS solution, i.e. time of lysis, three different times were tested, respectively 15, 30 and 60 s.

THP-1 cells were seeded and differentiated as described in II-1.2., and infected as described in II-1.3., in 96-well black (NUNC) and clear (Orange Scientific) plates. Since the use of clear plates for fluorimetric measurements are described by Jain et al. (2012), experiments were carried out on both clear and black plates to ensure that results were not significantly different in both plate types.

Control wells of non-infected cells were included in each experiment. Medium with SDS and without cells was used for background correction. Simultaneously, a 96-well clear plate was also included, to enable the observation of all the experiment steps under the inverted microscope.

After incubation period, the wells were washed 5 times with serum-free RPMI medium and 200 µL of RPMI at 2% FBS were added to each well. Plates were incubated for 48 h, to simulate the treatment with standard drugs or extracts. A solution of SDS (0.05 % *v/v* in serum-free RPMI) was prepared and 20 µL added to each well. After addition of the SDS solution, the plate was agitated for 15, 30 and 60 s, respectively. To inactivate the SDS, after the agitation time, 180 µL of RPMI at 10% FBS were added.

To each well, 20 µL of resazurin (250 µg mL^{-1}) were added and the plate was incubated for 24 h. The fluorescence was measured in a microplate flourimeter (Biotek

Synergy 4) at 550 and 590 nm as excitation and emission wavelengths, respectively. Results were expressed as percentage of viable cells, by comparison to the negative control cells, i.e., cells which were not subjected to lysis.

After lysis under the different times, both non-infected and infected cells were observed in an inverted microscope and images were acquired with a Canon EOS 500D camera to support the results obtained by fluorimetric measurements.

2.2.2. Evaluation of the effect of different parasite:cell ratio

To address if the method was able to discriminate different concentrations of released amastigotes, different parasite:cell ratios were applied in order to obtain increasing infection rates. Both the direct counting and parasite rescue methods were performed simultaneously.

Briefly, 16-well glass chamber slides were processed as described in II-1.3.2., and 96-well black plates were processed as explained in II-2.2.1., using different ratios and 30 s as the optimal lysis time.

A negative control of non-infected cells was included. Medium with SDS and without cells was used for background correction. RPMI medium at 10% with 20 µL of resazurin (250 µg mL^{-1}) were autoclaved for 15 min at 121ºC and used as positive control, resulting in a 100% reduced form of resazurin. Results were expressed as percentage of viability in comparison to non-infected cells, for 96-well plates. For 16-well glass chamber slides, results were expressed as percentage of infected cells and intensity of infection, as described previously in II-1.3.2.

2.2.3. Evaluation of the effect of the standard drug, Amphotericin B

For validation purposes, the effect of the standard drug AmB, was simultaneously evaluated using the parasite rescue method and the direct counting method. Briefly, 16-well glass chamber slides were processed as described in II-2.1.1. and 96-well black plates were processed as referred in II-2.2.1. using 30 s as the optimal lysis time.

A negative control of non-treated infected cells and a positive control for resazurin were included. The viability of amastigotes were obtained by comparison with the negative control wells, i.e. non-treated cells and the IC_{50} values (µg mL^{-1}) were determined.

3. Preparation of the extracts for antileishmanial screening

The macroalgae and halophyte material used in this study were provided by works in course at the MarBiotech Group.

3.1. Preparation of macroalgae and halophyte extracts

Concerning the macroalgae, all the samples belong to the *Cystoseira* genera, and were collected between July 2012 and March 2013 in different locations of the Iberian Peninsula (Table 4). Each sample was extracted with three solvents of increasing polarity, namely hexane (HEX), dichloromethane (DCM) and methanol, resulting in a total of 23 extracts. Only the *C. barbata* and the *C. nodicaulis* extracts were prepared in this work.

Table 4 Collection sites of the *Cystoseira* species used in this study

Species	**Collection site**
Cystoseira baccata	Areosa, Portugal
Cystoseira barbata	Cadiz Bay, Spain
Cystoseira humilis	Almograve, Portugal
Cystoseira nodicaulis	Santa Mariña, Spain
Cystoseira tamariscifolia	Areosa, Portugal
Cystoseira usneoides	Olhos de Água, Portugal

Dried biomass (4 g) was powdered and mixed with 40 mL of HEX, in triplicate. The rupture of algal cell walls was achieved with an IKA Ultra-Turrax disperser for 1 min. Samples were then homogenized (VWR VV3) and centrifuged 10 min at 5000 × *g*, at room temperature (RT). This procedure was repeated three times and then extracts were filtered with Whatman nº4 filter and combined. The residue was then sequentially extracted with dichloromethane and then methanol, filtered and concentrated under reduced vacuum pressure at 40ºC. Extracts were dissolved in DMSO (Merck) at a concentration of 50 mg mL^{-1} and stored at 4ºC.

Halophyte samples were collected in June 2013, in Ria Formosa, Portugal. A total of 26 species were included in this study for antileishmanial screening (Table 5). The aerial parts of each species were prepared and extracted with two solvents by stirring for

4 hours, namely acetone and dichloromethane, resulting in a total of 52 extracts. Extracts were filtered, concentrated under reduced vacuum pressure, ressuspended in DMSO at a final concentration of 25 mg mL^{-1} and stored at 4ºC for use in the bioactivity assays.

Table 5 Halophyte species used in this study.

Species	
Arthrocnemum macrostachyum	*Panicum repens*
Aster tripolium	*Pistacia lentiscus*
Calystegia soldanela	*Puccinelia* sp.
Centaurium erythraea	*Salicornia fragilis*
Cladium mariscus	*Salicornia ramosissima*
Frankenia laevis	*Salicornia perennis alpine*
Frankenia pulverulenta	*Salicornia perennis perennis*
Halopeplis amplexicaulis	*Spartina versicolor*
Inula crithmoides	*Spergularia rubra*
Lactuca sp.	*Sporobolus* sp
Limoniastrum monopetalum	*Salsola* sp.
Lythrum salicaria	*Salsola vermiculata*
Mesembrianthemum crystallinum	*Typha dominguensis*

In Soxhlet extraction, the dried biomass of *C. baccata* and *C. barbata* was taken into cellulose thimble in a ratio of biomass to solvent (1:6 *w/v*), placed in Soxhlet apparatus, refluxed for 8 hr until free of solvent colour, and it was using hexane:dichloromethane (HEX:DCM; 1:1). After extraction, solvent was removed using rotary evaporator under reduced vacuum pressure at 40ºC,, dissolved in DMSO at the concentration of 50 mg mL^{-1} and stored at 4°C.

4. Antileishmanial and cytotoxic activity of the extracts

4.1. Parasite and cell cultures

THP-1 cell line and *Leishmania infantum* strain MHOM/PT/88/IMT-151 were used in this study. Both cell and parasite cultures were maintained in RPMI medium as

described in II-1.1.

For viability assays, THP-1 and *L. infantum* promastigotes were maintained in RPMI medium for 3 days.

4.2. Evaluation of the effect of the extracts on axenic promastigotes

4.2.1. Determination of the promastigotes viability and 50% inhibitory concentration

Parasites viability was assessed on promastigotes forms of *L. infantum* by MTT assay. Briefly, the conversion of the tetrazolium salt MTT in the mitochondria of viable cells into purple formazan crystals, allows the quantification of viable cells, by spectrophotometric measurements (Van Meerloo et al., 2011).

The antileishmanial activity of a total of 52 extracts of halophytes and 23 extracts of the brown macroalgae was evaluated. In sterile clear 96-well plates, log phase promastigotes ($1,0 \times 10^7$ promastigotes mL^{-1}) were incubated with extracts at 125 µg mL^{-1}. Macroalgae and halophyte extracts were incubated for 48 and 72 h, respectively. Negative control cells were treated with DMSO at the highest concentration used in test wells (0,5 % *v/v*) and AmB, miltefosine and pentamidine were used as positive controls, at their previously determined IC_{50} values.

After incubation, 20 µL of MTT reagent (5 mg mL^{-1} in phosphate-buffered saline, PBS) were dispensed in each well and the plate was then incubated for 2 h at 36 ± 1 ºC at 5% CO_2. Since the parasites are in suspension, the plate was centrifuged for 15 min at 1479 × *g*, at 4 ºC and the supernatant was carefully removed. In order to dissolve the formazan crystals, 150 µL of DMSO were added per well. The absorbance was read in a microplate reader (Biotek Sinergy 4) at an emission wavelength of 590 nm, as described elsewhere (Rodrigues et al., 2014).

Extracts that inhibit the viability of parasites in more than 50 % at 125 µg mL^{-1}, were then tested, also by MTT assay, at seven different concentrations, ranging from 1.98 to 125 µg mL^{-1} and the IC_{50} values were determined.

4.2.2. Microscopy analysis of the promastigotes morphology

For microscopic analysis, parasites were incubated with the selected extracts for 48h, at 24 ± 1ºC, as described in II-4.2.1. After the end of the incubation period,

supernatants were centrifugated for 15 min at 1479 x *g*, at 4ºC, and the supernatant further discarded. The pellet was ressuspended in 20 µL of RPMI supplemented with 50% FBS and smeared on slides. After being dried and fixed with MeOH for 30 s, air-dried smears were stained with Giemsa (10% *v/v*; 10 min). After rinsed with H_2O_d and air-dried at RT, slides were directly covered with a slide and processed for microscopy analysis. Microscopic bright field images were acquired using a Zeiss AXIOMAGER Z2 microscope, equipped with a coolSNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) at the light microscopy facility of the Departmento de Medicina e Ciências Biomédicas of the Universidade do Algarve.

4.3. Evaluation of the effect of the extracts on mammalian cells

4.3.1. Determination of the THP-1 viability and IC_{50} concentration

Cytotoxicity was assessed on THP-1 cells by MTT colorimetric assay. Sterile 96-well plates were seeded with exponentially growing THP-1 cells at 1,0 x 10^5 cells mL^{-1}. PMA was added to the cell suspension at a final concentration of 25 ng mL^{-1} and 100 µL of the suspension were dispensed per well and incubated for 24 h to allow cells differentiation into adherent macrophages. The medium was then replaced by RPMI containing the macroalgae and halophytes extracts at 125 µg mL^{-1} and plates were incubated for 48 and 72 h, respectively. Control cells were treated with DMSO at the highest concentration used in test wells (0,5 % *v/v*) and cell viability was determined by the MTT assay, as described in II-4.2.1.

Extracts inhibiting the viability of THP-1 cells in more than 50 % at 125 µg mL^{-1}, being also active against *L. infantum* promastigotes, were then tested by the MTT assay, at seven different concentrations, ranging from 1.98 to 125 µg mL^{-1} and the IC_{50} values were determined.

4.3.2. Determination of the selectivity index

To evaluate the effectiveness of an extract against *L. infantum*, its selectivity index (SI) was calculated as follows:

$$SI = IC_{50}\,(THP\text{-}1)/\ IC_{50}\,(L.\ infantum)$$

where the numerator and the denominator represent the concentration of a extract required for 50% *in vitro* inhibition of THP-1 macrophages and the concentration of the same extract needed for 50% *in vitro* inhibition of *L. infantum* parasites, respectively. The selectivity index is a ratio that reflects the effectiveness of an extract, as discussed in I-3.

4.4. Evaluation of the effect of the extracts on the amastigote-macrophage model

The effect of the HEX:DCM extracts of *C. baccata* and *C. barbata*, obtained by hot Soxhlet-based extraction method, were evaluated towards *L. infantum* intracellular amastigotes. The citotoxicity of the extracts was previously evaluated as described in II-4.3.1.

The THP-1 cells were seeded and differentiated as described in II-1.2. and infected for 24h at a ratio of 10:1 as described in II-1.3.2. Six diluted suspensions of the extracts were prepared, ranging from 3.9 to 125µg mL^{-1}, obtained from serial dilutions (1:2) with RPMI medium at 2% FBS. Control cells were treated with DMSO at the highest concentration used in test wells (0.5 % *v/v*) were used as negative control. After 5 washes, 200 µL of each suspension was added to each well and the chamber-slide was incubated at 36ºC ± 1ºC in 5% CO_2 for 48 h. When the treatment period ended, the chamber was washed three times with serum-free RPMI, detached and stained with Giemsa. The percentage of infected cells and the intensity of infection were determined as described in II-1.3.2. The IC_{50} values were determined.

5. Statistical analysis and IC_{50} determination

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows. Analysis of variance (ANOVA) was used to assess differences between groups. When the null hyphotesis was accepted, multiple comparisons were performed to identify the statistically different groups at a significance level of 5% ($p < 0.05$), using the Tukey's and Dunnet's tests.

The IC_{50} values were calculated by sigmoidal fitting of the data in the GraphPad Prism version 5.0 software, using at least 5 concentrations.

Chapter III – Results and Discussion

1. Amastigote-macrophage model

1.1. Growth curve and relative percentage of metacyclic forms

To assess the growth dynamics of *L. infantum* promastigotes maintained in RPMI-1640 supplemented medium, daily counts of viable parasites were made for 9 days, without medium change. *L. infantum* presented low parasite densities, not exceeding 2 x 10^7 promastigotes mL^{-1} (Figure 6).

Metacyclogenesis occurs within the phlebotomine sand fly, in which parasites differentiate into infective promastigotes, named metacyclics. During this process, the parasite suffers morphological, structural and biochemical changes and also variations in gene expression (Saraiva et al., 1995; 2005). These alterations occur in axenic cultures, where a fraction of promastigotes from a logarithmic non-infective stage, which resemble procyclic promastigotes, are able to differentiate into stationary infective stage promastigotes, similar to their phlebotomine sand fly counterparts (Saraiva et al., 1995; 2005).

Giemsa smears were prepared and observed at day 3 and 5, representative of logarithmic phase and stationary phase, respectively (Figure 7). In general, at day 3 promastigotes presented rounded shape and short flagella. However, concerning the width of the cell body, mixed morphology was observed. The variability in the length of the cell body may be due to the fact that promastigotes are already in early stationary phase. The observed forms appear to be a mixture of procyclic and nectomonads, the latter being accepted as the second morphological stage in the midgut of the sand fly, more elongated than procyclic ones, as referred in I-1.1 (Kamhawi, 2006).

At day 5, promastigotes were highly motile and a high percentage of cells presented slender cell bodies in a characteristic needle shape with long flagella (flagellum > body length). These forms appeared to be leptomonads, the precursor forms of metacyclic promastigotes, which rapidly differentiate into the latter (Kamhawi, 2006; Kbaier-Hachemi et al., 2012). In fact, these observations were also carried out by Santarém et al. (2014) with *L. infantum* promastigotes cultivated in RPMI medium.

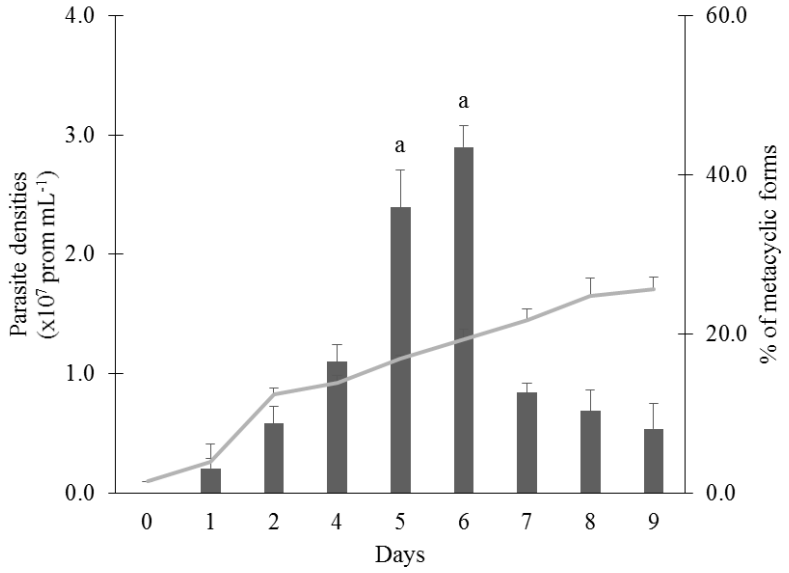

Figure 6 Growth dynamics of *L. infantum* IMT 151 axenic promastigotes maintained in RPMI. Light grey line represents the growth curve (promastigotes mL^{-1}); dark grey bars represent the percentage of metacyclic forms observed at each day. Both represent the mean ± SEM (standard error of the mean) of two independent experiments with three replicates each. a, no statistically differences between day 5 and 6, concerning % of metacyclic forms ($p > 0.05$; Tukey's test).

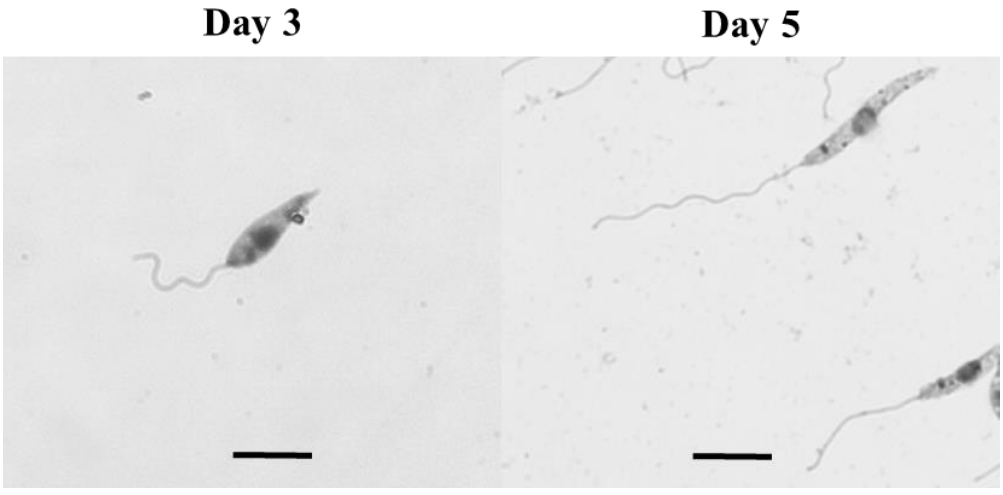

Figure 7 Predominant morphology of *L. infantum* axenic promastigotes cultured in RPMI medium. After 3 and 5 days of culture, promastigotes were smeared on slides and observed under a light microscopy. Bright field images, scale bar corresponds to 10 µm.

The distinction of metacyclic from procyclic parasites morphology is controversial. Some authors used to defend that there was no obvious morphological changes during metacyclogenesis (Da Silva and Sacks, 1987). However, recent studies on the purification of metacyclic promastigotes rely on clear morphological differences between the former and procyclic promastigotes (Spath and Beverly, 2001; Saraiva et al., 2005).

Morphological differences between parasites at the logarithmic and stationary phases were also observed in *L. infantum* promastigotes cultured in different mediums (Santarém et al., 2014). Thus, metacyclic promastigotes are generally accepted as small body cells with an elongated free flagellum, which is equal or greater than the body length and highly motile (Kamhawi, 2006; Bates, 2007).

Because of the subjectivity of optical microscopic counts, which can sub- or underestimate the number of metacyclic forms, other methods have been used by different authors in order to assess the metacyclogenesis process. For example, a clear overexpression of META-1 and SHERP genes at day 5, which are described as being express during metacyclogenesis, was observed for *L. infantum* promastigotes cultured in RPMI medium, supporting the results presented before (Moreira et al., 2012; Santarém et al., 2014). Furthermore, a method using negative peanut agglutinin (PNA) selection was shown to be as efficient to differentiate non-infectious (PNA^+) and infectious forms (PNA^-) of *L. infantum* promastigotes. The percentage of PNA^- forms were found at day 4 of culture, reaching a maximum of 80 %, when visceral strains were used (Kbaier-Hachemi et al., 2012).

Although the percentage of metacyclics may be overestimated in this study in comparison with other published works (Albuquerque et al., 2013), based on our results and those of others published on *L. infantum* (Maia et al., 2007; Moreira et al., 2012), a 5 day old culture of promastigotes was choosen for *in vitro* infection of macrophages, since no significant differences were observed between day 5 and 6 ($p > 0.05$). In future work, the purification of metacyclics might reduce the risk of infectivity variability, as *in vitro* infection of macrophages is dependent on the metacyclogenesis level.

1.2. Determination of the optimal parasite:cell ratio and infection period

To assess which parasite per macrophage ratio should be used for infection assays, five different ratios from 1.25:1 to 20:1 were applied. Two parameters were determined, namely the percentage of infected cells and the intensity of infection (Maia et al., 2007; Figures 8 and 9).

At 10:1 and 20:1, almost all macrophages became infected and the results obtained enable the determination of a dose-response curve (Figure 8). Statistically significant differences were observed between 5:1 and 10:1. However, no significant differences were observed between 10:1 and 20:1 ($p > 0,05$; Tukey's test) for the percentage of infected cells. As 50-70 % is thought as an adequate percentage of infected cells by some authors (Inocêncio da Luz et al., 2009), only 10:1 and 20:1 ratios were considered for *in vitro* infection.

Concerning the intensity of infection, no statistically significant differences were observed between 10:1 and 20:1 ratios ($p > 0.05$, Tukey's test; Figure 9). The ratio 10:1 was thus established as optimal for *in vitro* infection assays, since 20:1 did not enhance the results significantly, hampered the microscopic countings and high densities of promastigotes must be used for each assay. Several different ratios have been described for *in vitro* infections with *Leishmania* spp., the ratio chosen being within the range of those most commonly used in other studies (Inocêncio da Luz et al., 2009; Seifert et al., 2010; Christodoulou et al., 2011; Jain et al., 2012).

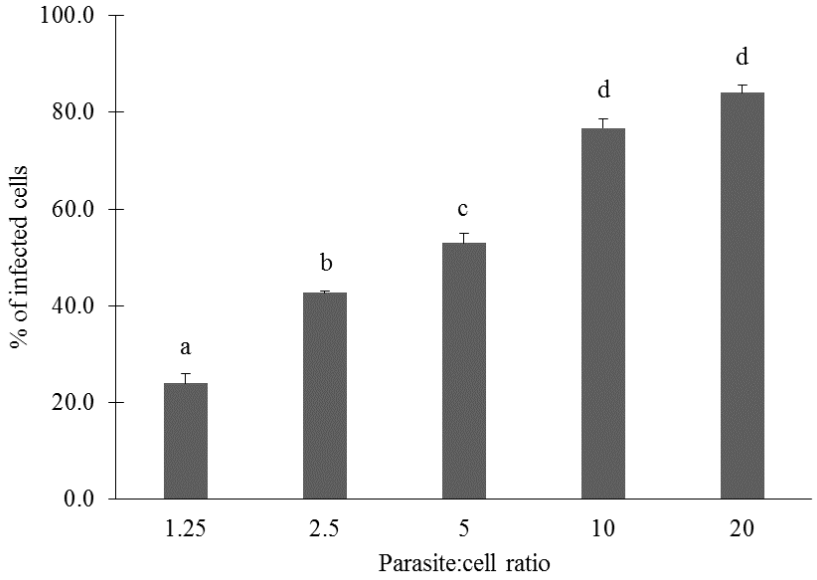

Figure 8 Percentage of infected cells obtained for different parasite:cell ratios. Bars represent the mean ± SEM, obtained from two independent experiments with two replicates each. Bars followed by different letters are significantly different ($p < 0.05$; Tukey's test).

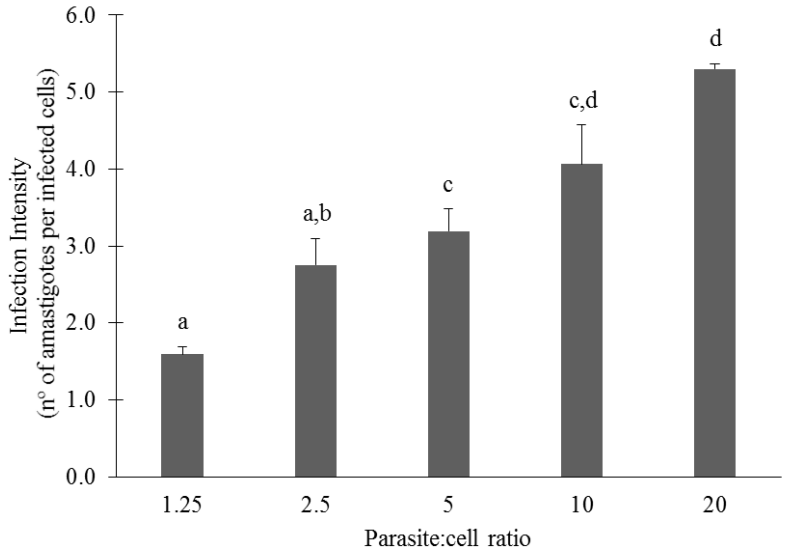

Figure 9 Intensity of infection obtained for each parasite:cell ratio applied. Bars represent the mean ± SEM, obtained from two independent experiments with two replicates each. Bars with different letters are significantly different ($p < 0.05$; Tukey's test).

It is described that short incubation periods were sufficient to obtain good infection rates (Seifert et al., 2010; Jain et al., 2012). In this context, three different incubation periods were tested, namely 4, 16 and 24 h (Figure 10). After 4 and 16h, no significantly differences were observed, and the percentage of infected cells reached a maximum of about 37%. After 24 h, about 70% of the cells became infected, as presented before (Figure 11).

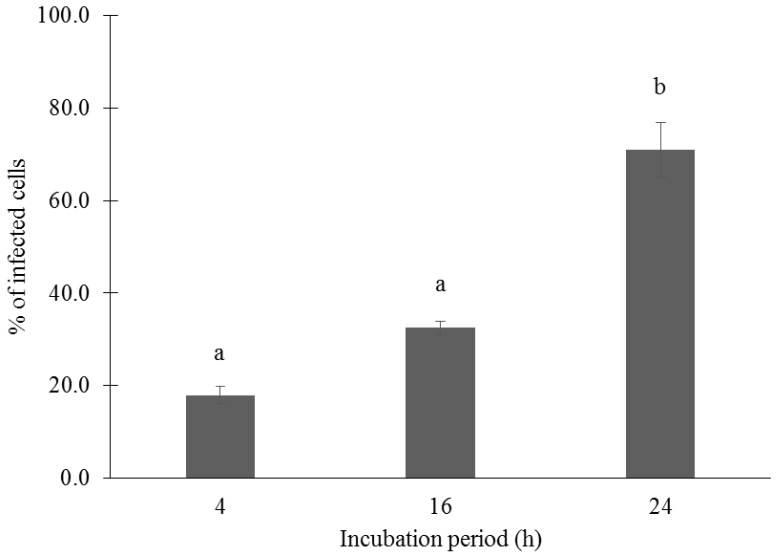

Figure 10 Percentage of infected cells after different incubation periods with 5 day old promastigote culture. Bars represent the mean ± SEM, obtained from two independent experiments with two replicates each. Bars with different letters are significantly different ($p < 0.05$; Tukey's test).

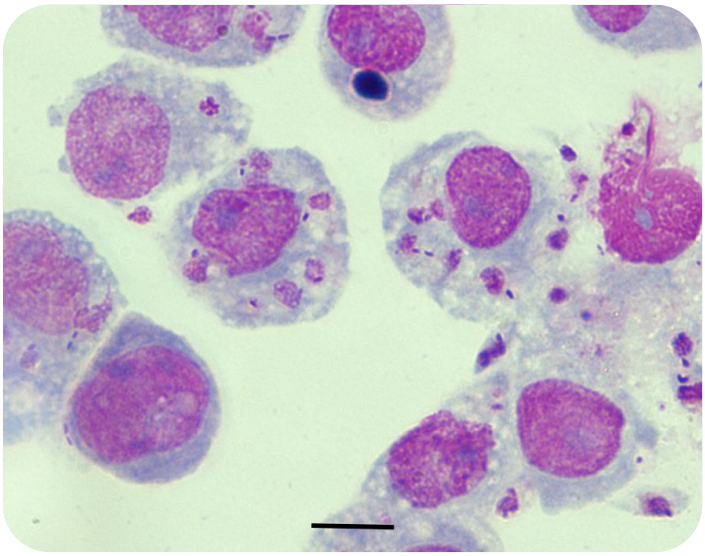

Figure 11 THP-1 derived macrophages infected with a 5 day old culture of metacyclic promastigotes of *L. infantum*, at a 10:1 infection ratio, for an infection period of 24 h (Original from Oliveira, M.). Scale bar corresponds to 10 µm.

Having in mind the results presented so far, the optimal conditions for the infection with *L. infantum* promastigotes were defined. Therefore, for infection assays, a 5 day old culture of *L. infantum* promastigotes was chosen for further assays of this study at a parasite:cell infection rate of 10:1 and subsequent incubation for 24 h in the presence of THP-1-derived macrophages.

2. Intracellular susceptibility assays

2.1. Direct counting method

AmB is a second-line drug widely used to treat leishmaniasis. It interacts with the ergosterol present in the parasite's membrane, leading to membrane disruption (Balaña-Fouce et al., 1998).

When comparing the IC_{50} values obtained for the promastigotes (IC_{50}, 0.6 ± 0.0 µg mL^{-1}) and the intracellular amastigotes (IC_{50}, 0.1 ± 0.0 µg mL^{-1}), it is clear that the second are more sensitive to the drug (Table 6). AmB didn't demonstrated cytotoxicity towards THP-1 cell line at the maximum concentration tested of 125 µg mL^{-1}.

Table 6 Antileishmanial activity against the promastigote and intracellular amastigote stages, cytotoxicity towards THP-1 cells and selectivity indexes, obtained for the standard drug, AmB.

	Promastigote	**Intracellular amastigotes**	**THP-1**
IC_{50} values ($\mu g\ mL^{-1}$)	0.6 ± 0.0	0.1 ± 0.0	>125
SI	>250	>1250	-
Method	MTT	Direct counting method	MTT

Values represent the mean ± SEM of at least three independent experiments performed in duplicate.

The different IC_{50} values obtained are reflected in the selectivity index, which increased five times for intracellular amastigotes. This fact is also referred by Vermeersch et al. (2009) who observed a higher specificity of AmB for the amastigote than for the promastigote stage of *L. donovani*. Moreover, when the susceptibility of *L. mexicana* promastigotes and intracellular amastigotes to six different antileishmanial drugs was compared, four of them presented different IC_{50} values for the two parasite stages (Callahan et al., 1997). Finally, this parasite stage-dependent specificity may be related to the mechanisms of action of the drugs or compounds, which reinforces the statement that promastigotes may not represent the ideal *in vitro* model for drug screening (Sereno et al., 2007).

2.2. Parasite rescue assay

2.2.1. Optimization of controlled cell lysis

After lysis with 0,05% SDS for 15, 30 and 60 s, a microscopic evaluation of the non-infected and infected wells was carried out in order to decide the ideal time of lysis (Figure 12). The optimal time should allow the lysis of the higher number of macrophages, slightly affecting the released amastigotes. Fifteen seconds were insufficient to achieve lysis of both non-infected as infected cells, once that a large number of macrophages was observed per field and consequently few intracellular amastigotes were released. After 30 s, even though some remaining macrophages were observed, the number of released amastigotes had increased considerably.

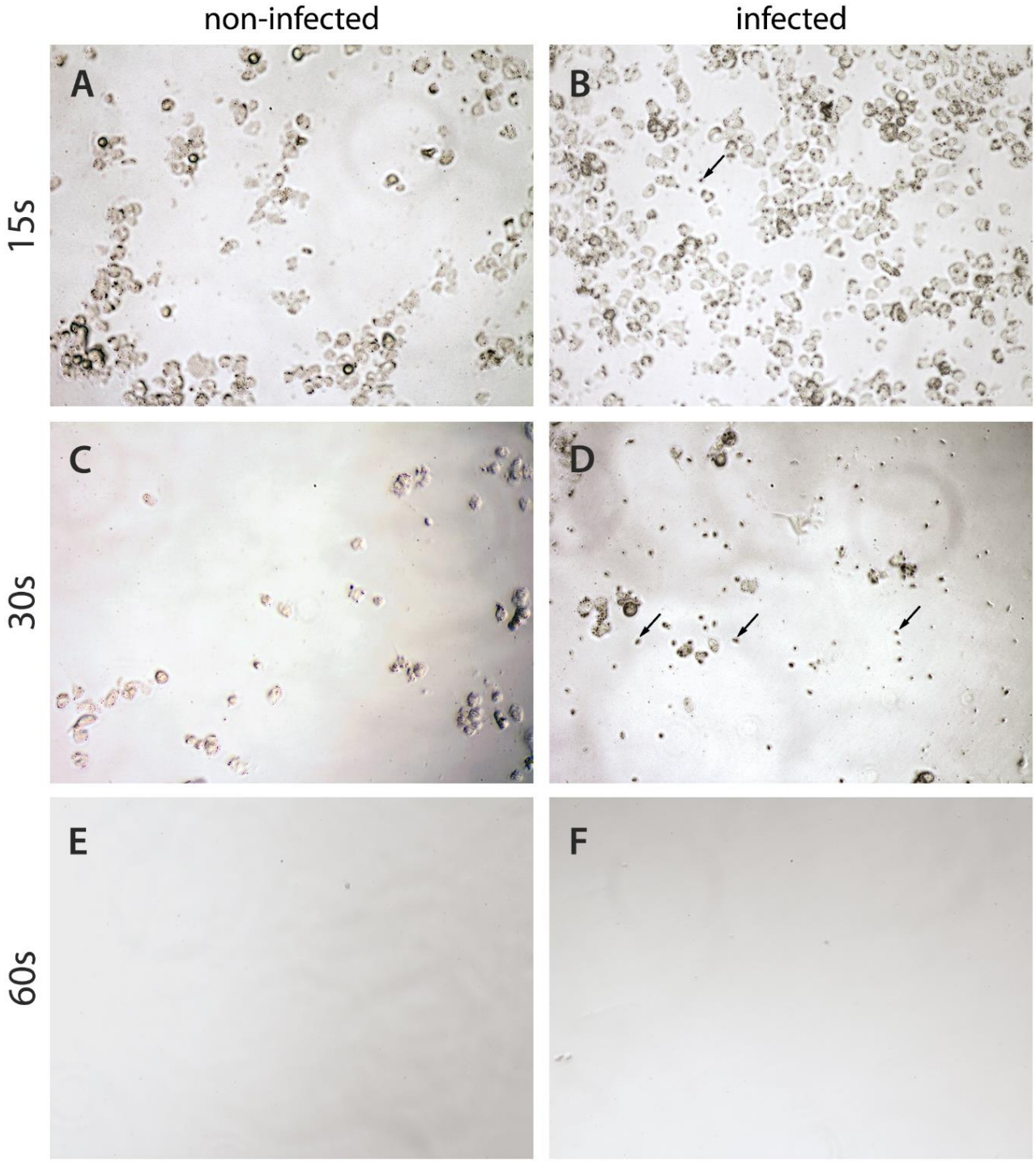

Figure 12 Microscopic evaluation of non-infected and infected macrophages at different times of lysis. A duration of 30 s was sufficient for quasi-complete lysis of macrophages with liberation of amastigotes (**arrows**). **N**on-infected macrophages lysed for 15 (**A**), 30 (**C**) and 60 s (**E**); infected macrophages lysed for 15 (**B**), 30 (**D**) and 60 s (**F**) (x 400).

After 60 s, macrophages or released amastigote forms were scarce in both infected and non-infected wells. These observations suggest that 60 s seems to be excessive, the SDS solution apparently affecting both cell lines. Taken together, the results of the microscopic evaluation allowed us to determine 30 s as the optimal lysis duration.

The measurement of the viability of *L. infantum* released amastigotes was assessed immediately after the lysis of infected cells, in both clear and black plates. The fluorescence signal of non-infected wells was also measured. Experiments performed in clear plates, after lysis of both infected and non-infected cells were not in accordance with the microscopic observations (Figure 13). In fact, results were very similar between all times tested and only a significant decrease in viability was observed after 60 s of plate agitation upon addition of SDS. Oddly, at 15 s, infected cells also presented a significant ($p < 0.05$) decrease.

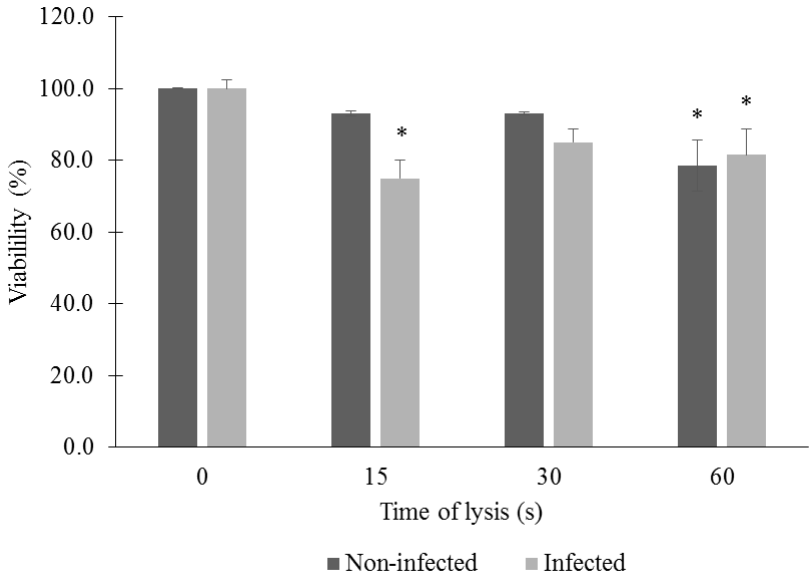

Figure 13 Viability of infected and non-infected cells in 96-well clear plates, non-lysed and lysed after 15, 30 and 60 s of plate agitation in the presence of SDS. Bars represent the mean ± SEM, obtained from two independent experiment with at least three replicates each. Statistically significant differences ($p < 0.05$; Dunnnet's test) in viability between control (i.e. non-lysed) and lysed cells at different times are marked with an *.

The disparity of these results can be attributed to the fact that clear plates are not recommended for fluorimetric measurements, although some authors use them in fluorescence based assays (Jain et al., 2012). Black plates present lower background, reduce well-to-well interference and minimize light scattering (Shekarchi et al., 1985; Corning Life Sciences, 2007). Therefore, the use of clear plates for this assay was abandoned.

Fluorescence measurements using black plates were somewhat different from those obtained for clear plates (Figure 14). Cells not subjected to lysis presented similar high fluorescence signals, with a slight increase in the arbitrary fluorescence units (AFU) in infected wells in comparison to non-infected (data not shown). Considering the minor difference between non-infected and infected cells and the fact that macrophages also metabolize resazurin, the need to lyse the cells was undoubtedly relevant to assess the effect of a drug on the intracellular parasites.

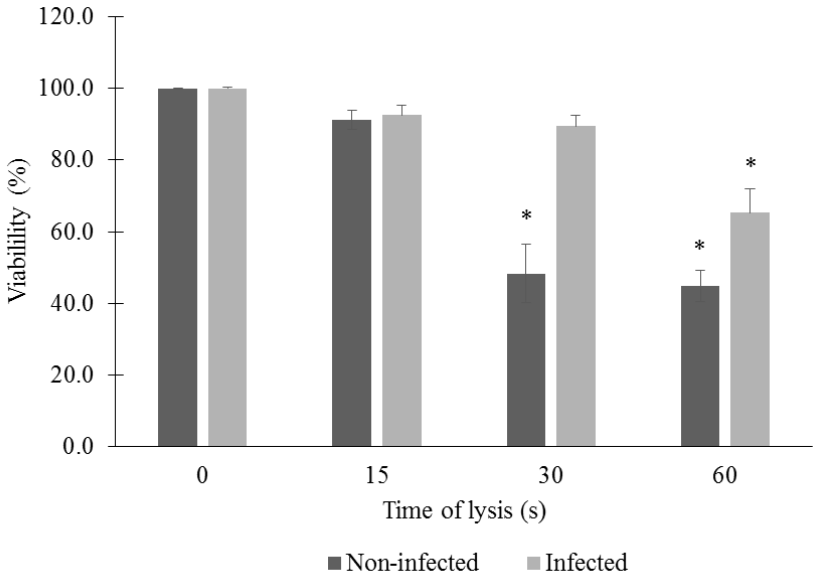

Figure 14 Viability of infected and non-infected cells in 96-well black plates, before and after 15, 30 and 60 s of plate agitation in the presence of SDS. Bars represent the mean ± SEM, obtained from two independent experiment with at least three replicates each. Statistically significant differences ($p < 0.05$; Dunnnet's test) in viability between control (i.e. non-lysed) and lysed cells at different times are marked with an *.

Although a small decrease in viability is evident after 15 s of agitation, a small positive difference between the viability for infected and non-infected wells can be verified. As observed in the microscopy images, few intracellular amastigotes were released and the majority of macrophages remained intact and metabolized resazurin.

After 30 s, the viability of infected cells (89.4 ± 2.6 %) remained unaltered when compared to that observed after 15 s (92.6 ± 2.6 %), while the AFU of the non-infected cells decreased significantly (48.3 ± 8.2%). This SDS agitation time induced the lysis of macrophages without affecting the viable amastigotes, confirming the observations at the inverted microscope. At 60 s an evident decrease in viability of both infected (65.3 ± 6.6%) and non-infected (44.8 ± 4.3%) cells was observed, suggesting that such exposure to SDS was excessive, resulting in lysis of both macrophages and released amastigotes. Although a viability of 44.8 % after 60 s of agitation was measured, few macrophages were observed in the digital images acquired. This is in agreement with the literature describing resazurin as being highly sensitive, since it can detect as low as 80-200 cells per well (O'Brien et al., 2000; Hamid et al., 2004; Borra et al., 2009).

Thus, for parasite rescue assay, 30 s were chosen as the optimal duration of SDS-induced cell lysis, because it promotes the lysis of almost all macrophages without significantly affecting the viability of the released amastigotes. Such a result is in accordance with previous studies (Jain et al., 2012). As the percentage of remaining macrophages after lysis was considered to be similar in all wells, only the infected wells were measured in further experiments.

2.2.2. Fluorimetric quantification of resofurin: pitfalls and challenges

The capability of the parasite rescue assay to discriminate different concentrations of released amastigotes was assessed by using 5 different parasite:cell ratios (Figure 15). Results were compared with the direct counting method, the classical approach optimized earlier (Figure 16).

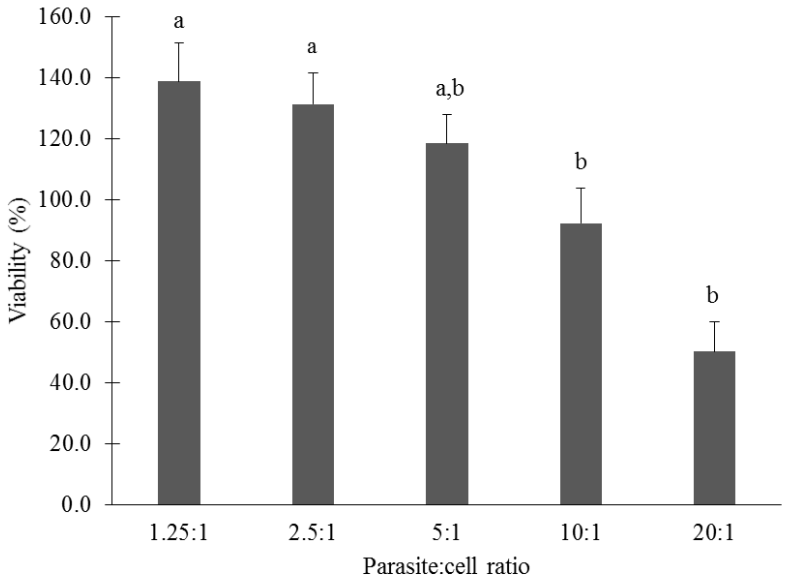

Figure 15 Viability obtained for infected cells with different ratios in 96-well black plates by means of the parasite rescue assay. Bars represent the mean ± SEM of two independent experiment with at least three replicates. Bars with different letters are significantly different ($p < 0.05$; Tukey HSD).

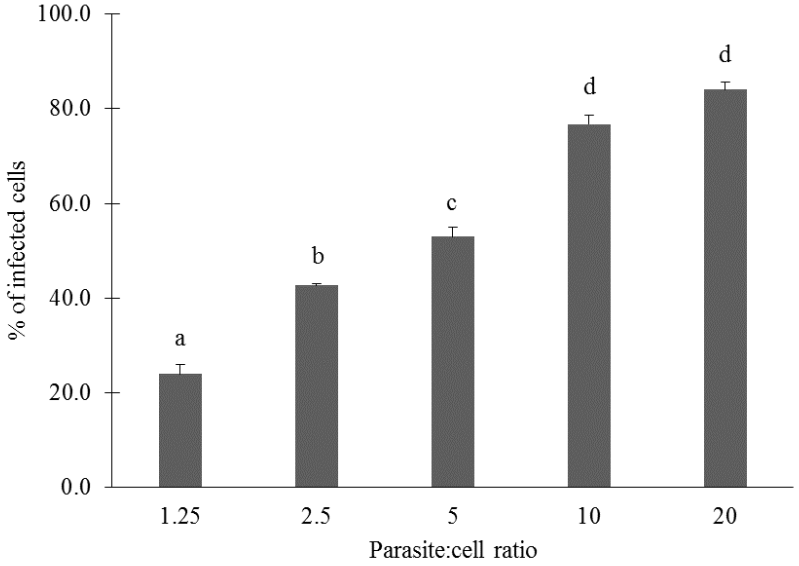

Figure 16 Percentage of infected cells obtained with different parasite:cell ratios in 16-well chamber slides by means of the direct counting method. Bars represent the mean ± SEM of two independent experiment with two replicates. Bars with different letters are significantly different ($p < 0.05$; Tukey's test).

Interestingly, the results obtained by the parasite rescue assay presented an inverted pattern in comparison to the results obtained by the direct counting method. As mentioned in I-3.3., resazurin is metabolized by living cells into the fluorescent product, resorufin. As a result, the viability of cells is directly proportional to the fluorescence produced. In this context, it would be expected that higher parasite:cell ratios applied should result in higher AFU due to increased cell counts, as observed in the direct counting method. However, the lower ratio presented the highest fluorescence values associated with higher viability. When AmB was tested as positive control, the same inverted pattern was detected, in which viability apparently decreased when lower concentrations of the drug were tested (Figure 17).

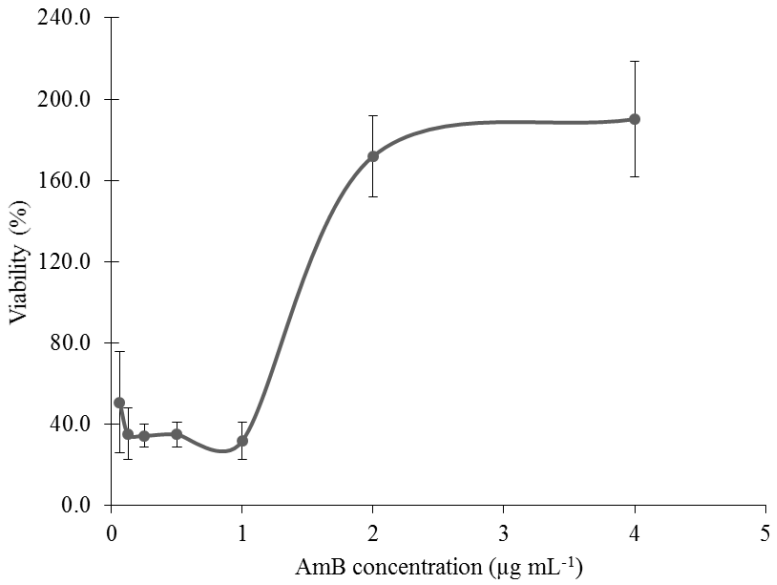

Figure 17 Effect of the standard drug AmB against intracellular amastigotes by means of the parasite rescue assay, with a parasite:cell ratio of 10:1. Bars represent the mean ± SEM of two independent experiments with three replicates each.

Resazurin is a blue non-fluorescent dye that has been widely used to assess the growth or inhibition of different types of cells. Despite its advantages, certain precautions should be considered during the optimization of resazurin-based methods, namely the cross reactivity of compounds (O'Brien et al., 2000; Stoddart, 2011). Thus, AmB was tested without cells to guarantee that it was not chemically reacting with resazurin producing a fluorescent product. Still, in experiments carried out with the different ratios, besides cells and parasites, no compound was added that would produce fluorescence when exposed to resazurin, and the inverted pattern persisted.

Moreover, the reduction rate of resazurin depends on the cellular line used which will further influence the resazurin concentration and incubation time optimization (O'Brien et al., 2000). This second step relies on the fact that the fluorescent product of resazurin, namely resorufin, can be further reduced into a colourless, non-fluorescent compound called dihydroresorufin (Figure 18; Rampersad, 2012).

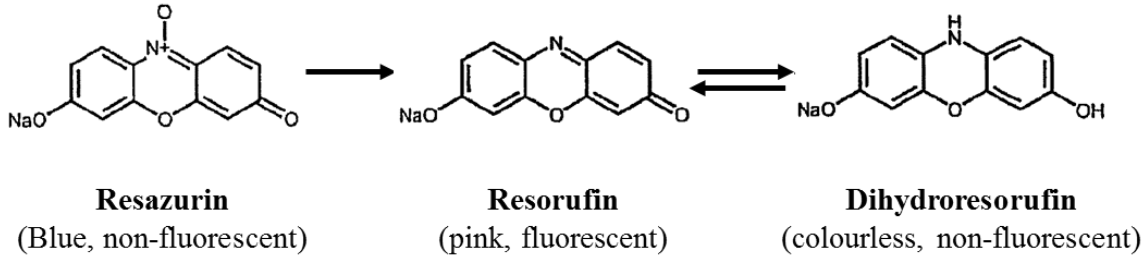

Figure 18 Metabolism of resazurin in mammalian cells (Fukushima et al., 2003).

This phenomena is known to occur in the presence of high cell densities or excessive incubation times (Rampersad, 2012). In this context, aberrant results may occur, as the living cells produce weak fluorescence signals due to the conversion into the non-fluorescent compound, and the dying cells produce high fluorescence because they can no longer further reduce resorufin (O'Brien et al., 2000). In fact, the characteristic metabolism of resorufin was already described as a useful tool to access the cytotoxicity on mammalian cells by long-term incubation with resazurin, giving more reliable results concerning cell death rather than being dependent on the number of viable cells (McMillian et al., 2002).

Having in mind the resorufin metabolism, it appears that this reduction probably influenced the results obtained during this study, assuming that at lower concentrations of AmB few amastigotes are present and vice-versa. Thus, the percentage of viability achieved may be the percentage of cellular death, due to excessive resazurin and resorufin reduction. Nonetheless, concerning the parasite:cell ratios experiment, it was expected that the higher the ratio used, increased infection rates were obtained, resulting in more released amastigotes, and thus increased fluorescence.

During this study, plates were incubated for 24 h. Most probably, if the incubation time was extended for additional 48 h, more consistent results would be obtained regarding cell death. In contrast, and more accurately, lower incubation times would give results comparable to those obtained by other viability assays, since the resorufin would not be reduced and the fluorescence signal would be associated to cell viability (McMillian et al., 2002).

Besides the incubation time, another factor relevant to this topic is the resazurin concentration used. Preliminary tests using resazurin in the MarBiotech group were carried out at 2.5 µg per well, because it is the most commonly used concentration for viability assays using promastigotes of *Leishmania* (Spavieri et al., 2010a). However, at this concentration, no significant fluorescence signals were obtained. Hence, for this study, the concentration of resazurin was increased to 6.25 µg per well with further incubation for 24 h , which was also previously described for promastigotes (Kulshrestha et al., 2013).

Nevertheless, the parasite forms metabolizing resazurin used in this study were the released amastigotes, which are comparable to axenic amastigotes. Resazurin has also been used to assess the viability of axenic amastigotes in drug screening assays (Spavieri et al., 2010a).

Curiously, the axenic amastigotes were found to attain the fluorescence peak faster than promastigotes, with further decrease in fluorescence at elevated parasite densities and excessive incubation times (Shimony and Jaffe, 2008). These differences observed between the reduction rates of promastigotes and amastigotes have been suggested to be caused by different incubation temperatures and atmospheres, since the latter are maintained at 36ºC with 5% CO_2 and the former at 25ºC (Mikus and Steverding, 2000).

In fact, a resazurin-based method applied to evaluating cell growth and inhibition of *Leishmania* promastigotes yielded more consistent results when plates were incubated in 5% CO_2 atmosphere or reduced air availability, allowing the efficient reduction of resazurin (O'Brien et al., 2000; Corral et al., 2013).

As discussed before, the remaining macrophages in the wells may also have contributed to the aberrant results shown in figures 15-17, by enhancing fluorescence quenching through metabolization of the resazurin and, consequently, the resorufin compound. Again, this could have been avoided if different conditions were applied. Therefore, the number of remaining cells results in a background signal in all wells.

In summary, and having in mind all the results discussed in this section, the resazurin concentration and incubation time used in this study was shown to be inadequate for the assessment of the viability of the released amastigotes. In future experiments, both lower incubation times and resazurin concentrations should be tested to completely optimize the assay.

Although resazurin-based methods are undoubtedly simple, fast, less expensive and pointed out as giving accurate results, the optimization process may be long and difficult, even more so when two different types of cells are used, in this case the mammalian cellular line THP-1 and *L. infantum* parasites (O'Brien et al., 2000).

As argued above, the parasite rescue assay relies on the success of several critical steps such as the differentiation of cells into adherent macrophages, infection with *L. infantum* promastigotes, lysis of infected cells and fluorimetric quantification after resazurin. Just a trivial failure in one of these steps or a problem with the cell cultures would compromise the entire assay, which can take up to one week.

The advantages of optimizing this assay for drug screening purposes are undeniable, compared with the classical methodology, which is laborious and often discarded for screening of large numbers of extracts, as referred in I-3.2.. Unfortunately, as other methodologies focusing on intracellular amastigotes, the inherited difficulties of this model in high-throughput screenings are highlighted, and our objective of optimization of a high-throughput assay was not achieved.

3. Antileishmanial activity and cytoxicity of halophytic and algal extracts

3.1. Halophytic extracts

In this work aqueous acetone and dichloromethane extracts from 26 halophyte species were evaluated for their *in vitro* activity against the promastigote stage of *L. infantum* and cytotoxic effect on THP-1 cells. Extracts were applied for 72 h at a concentration of 125 µg mL^{-1} and results are summarized in Table 7.

Regarding ACE extracts, only that of *Calystegia soldanella* (syn. *Convolvulus soldanella*) brought about a significant reduction in promastigote viability (down to 57%). However, this extract was not selective towards promastigotes, since it also reduced the viability of differentiated THP-1 cells (down to 37%). Dichloromethane (DCM) extracts were more active towards both cell line and parasites. The application of the dichloromethane extracts of *I. crithmoides* and *Spergularia rubra* resulted in the lowest promastigotes viability (27 % and 17 % with IC_{50} values of 59.5 ± 0.9 µg mL^{-1} and 70.5 ± 1.7 µg mL^{-1}, respectively). However, these extracts were also highly cytotoxic to differentiated THP-1 cells: that of *I. crithmoides* and of *S. rubra* displaying an IC_{50} of 20.3 ± 0.3 µg mL^{-1} (SI=0.3) and 49.3 ± 0.4 µg mL^{-1} (SI=0.7), respectively.

Moreover, the DCM extracts of *L. salicaria, Salsola sp., L. monopetalum, S. perennis alpini, S. ramosissima* and *S. vermiculata* all presented an interesting feature: they all modestely decreased the viability of promastigotes without significantly affecting the viability of THP-1 cells.

Table 7 Antileishmanial activity and cytotoxicity towards THP-1 cells of acetone and dichloromethane extracts of different halophyte species.

Species	**Extract**	**Viability** (%)	
		L. infantum	THP-1
Arthrocnemum macrostachyum	ACE	105.9 ± 1.5	114.1 ± 2.9
	DCM	79.2 ± 0.3*	14.1 ± 0.6*
Aster tripolium	ACE	108.6 ± 0.9	81.0 ± 3.4*
	DCM	94.8 ± 0.5	72.4 ± 4.6*
Calystegia soldanella	ACE	57.8 ± 4.7*	36.6 ± 0.9*
	DCM	75.5 ± 7.7*	51.5 ± 2.1*
Centaurium erythraea	ACE	87.2 ± 3.5	86.9 ± 3.3
	DCM	89.3 ± 1.6	62.3 ± 1.8*
Cladium mariscus	ACE	114.7 ± 5.1	126.7 ± 2.7*
	DCM	92.6 ± 1.1	101.9 ± 2.8
Frankenia laevis	ACE	126.4 ± 2.7*	176.5 ± 2.7*
	DCM	107.2 ± 2.2	34.1 ± 0.5*
Frankenia pulverulenta	ACE	137.1 ± 3.1*	142.2 ± 6.1*
	DCM	88.9 ± 3.8	76.5 ± 1.4*
Halopeplis amplexicaulis	ACE	90.4 ± 1.7	120.4 ± 5.5*
	DCM	73.1 ± 6.4*	45.3 ± 1.9*
Inula crithmoides	ACE	88.9 ± 0.8	37.8 ± 1.6*
	DCM	26.5 ± 0.7*	10.5 ± 0.3*
Lactuca sp.	ACE	102.5 ± 6.5	122.7 ± 4.4*
	DCM	82.8 ± 1.7*	100.5 ± 3.1
Limoniastrum monopetalum	ACE	117.6 ± 3.2*	147.1 ± 2.4*
	DCM	82.2 ± 0.8*	101.3 ± 2.1
Lythrum salicaria	ACE	143.2 ± 1.7*	84.3 ± 2.9
	DCM	76.0 ± 3.1*	114.0 ± 2.0*
Mesembrianthemum cristalino	ACE	106.1 ± 9.9	134.6 ± 4.7*
	DCM	85.4 ± 0.7*	132.1 ± 3.8*
Panicum repens	ACE	92.7 ± 4.0	120.9 ± 2.9*
	DCM	97.6 ± 2.9	52.5 ± 0.2*
Pistacia lentiscus	ACE	130.6 ± 2.5*	130.6 ± 5.8*
	DCM	89.6 ± 1.9	31.4 ± 0.2*
Puccinelia sp.	ACE	88.2 ± 1.8	122.8 ± 5.4*
	DCM	90.2 ± 0.9	111.0 ± 2.9*
Salicornia fragilis	ACE	100.0 ± 1.9	151.2 ± 2.9*
	DCM	78.4 ± 4.6*	75.8 ± 1.8*

Table 7 Cont.

Species	**Extract**	**Viability** (%)	
		L. infantum	THP-1
Salicornia ramosíssima	ACE	92.0 ± 1.7	151.3 ± 7.6*
	DCM	78.8 ± 1.1*	107.0 ± 2.2
Salicornia perennis alpini	ACE	97.3 ± 1.6	114.5 ± 6.1
	DCM	78.2 ± 3.0*	119.7 ± 1.0*
Salicornia perennis perennis	ACE	97.1 ± 1.8	143.4 ± 7.3*
	DCM	79.3 ± 1.7*	13.7 ± 0.5*
Salsola sp.	ACE	90.7 ± 2.1	79.7 ± 2.1*
	DCM	84.6 ± 1.2*	121.3 ± 3.3*
Salsola vermiculata	ACE	100.3 ± 0.7	108.2 ± 5.7
	DCM	64.3 ± 5.3*	105.3 ± 1.6
Spartina versicolor	ACE	115.0 ± 1.4*	122.8 ± 1.6*
	DCM	92.4 ± 1.3	125.4 ± 3.0*
Spergularia rubra	ACE	89.7 ± 2.7	107.2 ± 4.8
	DCM	16.7 ± 0.5*	29.6 ± 0.2*
Sporobolus sp	ACE	100.0 ± 2.9	81.7 ± 3.1
	DCM	98.9 ± 2.3	96.8 ± 3.1
Typha dominguensis	ACE	94.9 ± 1.4	134.8 ± 2.7*
	DCM	91.2 ± 0.8	83.9 ± 4.0*
DMSO		99.8 ± 1.2	100.0 ± 0.2
Amphotericin B[1]		n.d.	67.3 ± 4.2
Pentamidine[1]		n.d.	12.2 ± 0.1
Miltefosine[1]		n.d.	8.0 ± 0.1

Values represent the mean ± SEM, obtained from at least three independent experiments performed in triplicate. n.d. not determined; [1]Positive controls; Statistical significance in viability between cells containing DMSO (0.5%, *v/v*) diluted in culture medium and those treated with extracts are indicated as * ($p < 0.05$; Dunnet's test).

C. *soldanella* also known as the morning glory and the Prince's Flower is a perennial plants that can be found in coastal habitats in temperate regions of the world, and has been used for centuries in traditional medicine as a purgative (Pereda-Miranda et al., 2010; Takigawa et al., 2011). It belongs to the *Convolvulaceae* family, which is known for biosynthetizing unique secondary metabolites namely resin glycosides (Tasdemir et al., 2008). In fact, hapargide and crypthophilic acid C, purified from a MeOH extract of the aerial parts of *Scrophularia cryptophila,* had high activity towards *L. donovani* axenic amastigotes (Tasdemir et al., 2008).

It is not surprising that the ACE extracts were poorly active as some authors claim that the bioactive compounds towards *Leishmania* are mainly found in less polar extracts (Genovese et al., 2009; Vonthron-Sénécheau et al., 2011). However, extraction with 80% ACE is recommended to extract phenolics, since some studies have put forward the antileishmanial potential of this group of compounds (Zhao et al., 2006; De Arias et al., 2012).

The genus *Inula* is widely distributed through Africa, Asia and Europe, especially in the Mediterranean area. At least 100 species are known and have been extensively used in Chinese traditional medicine (Seca et al., 2014). Interestingly, flowers from *I. crithmoides* have been used in traditional medicine to treat several urinary related illnesses, but also infectious diseases, such as malaria and tuberculosis (revised by Dos Santos, 2009). More recently, mainly due to their ethnopharmacological relevance, the chemotherapeutic properties of various *Inula* species have been investigated, which were found to possess cytotoxic, antibacterial, antioxidant and hepatoprotective activities (Zhao et al., 2006; Giampieri et al., 2010).

Plants belonging to the *Spergularia* genus are found in temperate and subtropical regions of all continents, except Antartida. Although they have been used in traditional medicine of Kuwaiti and Mediterranean region as a remedy for kidney stones, other related kidney illnesses, and as a diuretic (Middleditch and Amer, 1991; González-Tejero et al., 2008), few biological activities have been reported for *S. rubra*. However, water extracts obtained from infusion of the whole plant have significantly reduced the glucose blood levels in experiments with diabetic rats (Bnouham et al., 2002) and its aerial parts have antiradical, antidiabetic and anticholinesterase activities (Vinholes et al.,2011).

So far, to our knowledge, there is no report on the antileishmanial activity or cytotoxicity for *S. rubra* and *I. crithmoides*. Moreover, to the best of our knowledge, there are no reports of the antileishmanial activity in halophytes.

According to Cos et al. (2006) natural extracts for anti-infective assays are considered relevant when they exhibit IC_{50} values lower than 100 μg mL^{-1}, taking the selectivity index into account (Cos et al., 2006). In this study, besides its good antileishmanial activity, the high cytotoxicity observed makes these extracts unappealing for further investigations, since the selectivity indexes obtained are low (<1; Table 8).

Table 8 Antileishmanial activity (promastigotes), cytotoxicity (IC_{50} values) and selectivity index (SI) of acetone and dichloromethane extracts of *S. rubra* and *I. crithmoides*.

Species	**Extract**	**IC_{50} values** (μg mL^{-1})		**SI**
		L. infantum	THP-1	
I. crithmoides	ACE	>125	n.d.	n.d.
	DCM	59.5 ± 0.9[a]	20.3 ± 0.3[a]	0.3
S. rubra	ACE	>125	>125	n.d.
	DCM	70.5 ± 1.7[b]	49.3 ± 0.4[b]	0.7
Amphotericin B		0.54 ± 0.0[c]	>125	>231.5
Miltefosine		14.6 ± 0.6[d]	7.7 ± 0.1[c]	0.5
Pentamidine		0.81 ± 0.0[c]	52.7 ± 1.9[b]	65.1

n.d., not determined; Values represent the mean ± SEM of three independent experiments performed in triplicate. Values followed by different letters are significantly different ($p < 0.05$; Tukey's test).

However, it is worth mentioning that the SI of the DCM extract of *S. rubra* (0.7) was similar to that of miltefosine (0.5), an established oral drug used in leishmaniasis treatment and as positive control in this study. In contrast, it was substantially lower than that of the other positive controls, namely AmB and pentamidine.

It is important to emphasize the relative significance of the cytotoxicity values and the selectivity indexes obtained. *S. rubra* and *I. crithmoides* were poorly selective. However, it may be considered that an extract is a mixture of compounds. Thus, it is difficult to discriminate, without a bioguided fractionation study, if the high cytotoxicity and antileishmanial activities observed for the same extract are produced by the same or different compound(s) present (De Toledo et al., 2014). Thus, further research on this species potential must be considered, because the bioactive compounds may in fact be selective upon fractionation.

Despite the low activity towards promastigotes and/or high toxicity on differentiated THP1 cells, additional studies should be performed in order to fully explore the antiparasitic potential of the halophytes species under study.

Firstly, in this work, only two solvents were used, namely acetone and dichloromethane. Since an extract is a mixture of compounds and considering that there is no solvent that will isolate all its constituents, perhaps the compounds of interest were not extracted by the solvents used, or were only present in low concentrations in the

mixture. For example, the *S. rubra* acetone extract did not present any antileishmanial or cytotoxicity, although the DCM extract was significantly active for both the parasites and mammalian cells. Most probably, in this case, the bioactive compounds are not soluble in ACE, but in DCM. Thus, in future work, other solvent systems may be tried to evaluate the antileishmanial activity of these species.

Secondly, only the aerial parts of halophytes were extracted in this study. As the roots of halophytes have already been described as sources of bioactive compounds, these may be included in additional screening efforts (Rasheed et al., 2013).

Lastly, cold extraction was used in this study. However, previous results obtained by our group indicate that the extraction method may influence the isolation of compounds with antileishmanial activity. In future studies, hot extractions may be performed in order to collect higher ammounts of the compound(s) of interest, since these may be lost in the process. In fact, it was already observed significant variations in the percentage of yield of the compounds of interest obtained between plant species extracted with the same solvent, between solvents used for the same species, and also between the extraction methods applied, which affects the overall bioactivities tested (Bouaziz et al., 2009).

Moreover, alternative studies with the non-active and non-toxic extracts could also be performed. Although inactive towards both cellular lines, these may be able to modulate the macrophages response preventing *L. infantum* promastigotes infection by increasing reactive oxygen species and nitric oxide production, for instance. As *Leishmania* parasites are able to evade the immune defense arsenal by inhibiting ROS and NO production and increasing the activity of suppressive cytokines, extracts enhancing the response of macrophages may be useful in the fight against infection (Shio and Olivier, 2010). After infection with *L. amazonensis* promastigotes of pre-treated macrophages with the crude aqueous extract of the palm tree *Syagrus coronate* (33 µg mL^{-1}), a decrease of 70.4% in the association index (number of parasites that successfully infected the macrophages) as well as an increase of 158.3% of NO production, in comparison to untreated cells, was observed (Rodrigues et al., 2011). Likewise, after infection with *L. amazonensis* of pre-treated macrophages with linalool-rich essential oil extracted from *Croton cajucara* (15 ng mL^{-1}), the association index was 50% lower and NO production was 220% higher (Rosa et al., 2003). Also, the pre-incubation of

macrophages with *Aloe vera* leaf exudate has led to activation of macrophages through increased ROS production, which enhanced the antileishmanial activity of the exudate (Dutta et al., 2007).

Interestingly, Arevalo et al. (2001) have shown that Imiquimod, acts as a modulator of the immune response by increasing the production of cytokines such as IFN-γ and interleukines such as TNF. Moreover, *in vitro* studies revelead its capacity to eliminate *Leishmania* amastigotes by increasing the production of NO. Furthermore, when a combined imiquimod plus meglumine antimonate therapy was applied to 12 patients with CL, whom were not responding to meglumine antimonate therapy earlier, 90% of those were cured after 6 months (Arevalo et al., 2001).

Taken together, these studies suggest that the study of the macrophage activation may be an alternative pathway in the search for bioactive substances with pharmacological properties, which may be useful tools in the battle against leishmaniasis.

3.2. Algal extracts

Concerning the antileishmanial activity of macroalgae from the *Cystoseira* genus, 21% of the extracts tested were active towards the promastigotes of *L. infantum*. The most interesting extracts were the HEX and DCM extracts of *C. tamariscifolia* and the DCM extract of *C. usneoides* with IC_{50} values of 31.2 ± 0.9, 29.8 ± 0.5 and 33.6 ± 0.6 µg mL^{-1}, respectively. Yet, their high cytotoxicity towards the mammalian cell line makes both extracts poorly selective (SI of 1, 0.7 and 0.4, respectively).

Moreover, the HEX extracts of *C. baccata* and *C. barbata* displayed moderate activities, with IC_{50} values of 94.1 ± 1.5 µg mL^{-1} and 78.7 ± 3.2 µg mL^{-1}, respectively (Table 9). However, the HEX extract of *C. barbata* also revealed mild cytotoxicity, with an IC_{50} value of 60.9 ± 2.0 µg mL^{-1} and consequently, the SI value was lower than 1. In summary, the HEX extract of *C. baccata* was the most selective extract. Since the maximum concentration tested was 125 µg mL^{-1}, the SI is higher than 1.32.

The cytotoxicity of the macroalga *C. tamariscifolia* is extensively described, using solvents of different polarities (Abourriche et al., 1999; Ainane et al., 2014). Likewise, *C. tamariscifolia* is a proven rich source of anti-proliferative compounds. Hence, the activity of its extracts has been previously demonstrated towards Daudi, Jurkat, K562, SUP-T1, PF-382 and THP-1 cell lines (Zubia et al., 2009; Vizetto-Duarte et al., 2012).

At 125 µg mL^{-1}, the HEX and ether extracts of *C. tamariscifolia* decreased the viability of THP-1 cells to 14.3±0.6 and 14.9 ± 0.9 µg mL^{-1}, respectively (Vizetto-Duarte et al., 2012). In our study, at this concentration the HEX and DCM extracts of this species decreased the viability of THP-1 macrophages to 18.6 ± 0.6 and 17.0 ± 0.5%, respectively (data not shown). Thus, the results obtained are in agreement with the published works mentioned above.

The antileishmanial activity of *C. tamariscifolia* and *C. baccata* collected in the south coast of United Kingdom were evaluated using axenic amastigotes of *L. donovani*, and both presented promising IC_{50} values, below 10 µg mL^{-1} (Spavieri et al., 2010). However, the ether and chloroform extracts of *C. tamariscifolia* collected in Morocco, presented IC_{50} values towards *L. infantum* promastigotes above 100 µg mL^{-1} (Ainane et al., 2014). Likewise, the hexane and ether extracts of *C. baccata* from the Portuguese coast, exhibited remarkable IC_{50} values against *L. infantum* promastigotes with IC_{50} below 8 µg mL^{-1} (Bruno de Sousa et al., 2012).

Interestingly, the MeOH extracts of *C. barbata* collected from the Black Sea and the Marmara Sea displayed different antileishmanial activities towards *L. donovani* amastigotes with IC_{50} values of 23.46 and 69.98 µg mL^{-1}, respectively (Süzgeç-Selçuk et al., 2010). No cytotoxicity against L6 cells were observed at the maximum concentration tested of 90 µg mL^{-1} (Süzgeç-Selçuk et al., 2010).

In this study, the HEX and DCM extracts of *C. tamariscifolia* presented high antileishmanial activity towards *L. infantum* promastigotes, with IC_{50} values below 35 µg mL^{-1}, while the HEX extracts of *C. barbata* and *C. baccata* presented IC_{50} values between 75-95 µg mL^{-1}. Undoubtedly, these 3 species are endowed with compounds able to kill *Leishmania* parasites, as confirmed by others authors.

Table 9 Antileishmanial activity and cytotoxicity (IC_{50}, μg mL^{-1}) of extracts of macroalgae from the *Cystoseira* genus.

***Cystoseira* species**	**Extracts/ compound**	**IC_{50} values** (μg mL^{-1})		**SI**
		L. infantum	THP-1	
C. baccata	Hex	94.1 ± 1.5[a]	>125	>1.32
	DCM	>125	n.d.	-
	$MeOH_1$	>125	>125	-
	$MeOH_2$	>125	>125	-
C. barbata	Hex	78.7 ± 3.2[b]	60.9 ± 2.0[a]	0.8
	DCM	>125	n.d.	-
	MeOH	>125	>125	-
C. humilis	Hex	>125	>125	-
	DCM	>125	>125	-
	$MeOH_1$	>125	>125	-
	$MeOH_2$	>125	>125	-
C. nodicaulis	Hex	>125	n.d.	-
	DCM	>125	>125	-
	$MeOH_1$	>125	>125	-
	$MeOH_2$	>125	>125	-
C. tamarascifolia	Hex	31.2 ± 0.9[c]	30.9 ± 0.4[b]	1
	DCM	29.8 ± 0.5[c]	19.9 ± 0.5[c]	0.7
	$MeOH_1$	>125	n.d.	-
	$MeOH_2$	>125	>125	-
C. usneoides	Hex	>125	n.d.	-
	DCM	33.6 ± 0.6[c]	12.6 ± 0.4[d]	0.4
	$MeOH_1$	>125	n.d.	-
	$MeOH_2$	>125	>125	-
Amphotericin B		0.2 ± 0.0[d]	>125	>625
Miltefosine		12.7 ± 1.8[e]	9.0 ± 0.5[d]	0.7
Pentamidine		0.5 ± 0.1[d]	68.2 ± 1.5[e]	136.4

n.d., not determined; Values represent the mean ± SEM of three independent experiments performed at least in duplicate**.** Values followed by different letters are significantly different ($p < 0.05$; Tukey's test).

Previous studies focusing on the chemical bioactive molecules from species belonging to the *Cystoseira* genus have identified mainly diterpenoids, meroditerpenoids, phlorotannins and sterols (Mokrini et al., 2008; Mighri et al., 2009). Mainly meroditerpenoids have been isolated from *C. baccata* (Mokrini et al, 2008), halogenated hydrocarbons and sterols from *C. barbata* (Milkova et al., 1997) and phloroglucintriacetates, phlorotannins and meroditerpenoids from *C. tamariscifolia* (Bennamara et al., 1999; Lopes et al., 2012). The latter two were also tested for antifungal

and antibacterial activities. However, no studies reporting the antileishmanial activity of compounds isolated from these species have been found in the literature.

The dispair antileishmanial activities observed between the activity of the crude extracts of our study and others for this three macroalgae, may be related to the different *Leishmania* species or strains, parasite stages, solvents used and extraction methods applied. For example, a diterpene compound isolated from the macroalga *Canistrocarpus cervicornis* exhibited an IC_{50} value of 12.0 μg mL^{-1} towards the axenic amastigotes of *L. amazonensis*. Still, towards the promastigote and intracellular amastigote forms the IC_{50} values decreased to 2.0 μg mL^{-1} and 4.0 μg mL^{-1}, respectively (Dos Santos et al., 2010). Moreover, the location and period of the biomass collection influences quality and the quantity of the secondary metabolites present in it, resulting in differences in the bioactivities obtained (Spavieri et al. 2010b; Vonthron-Sénécheau et al. 2011). As an example, *C. barbata* collected in two different locations provided different antileishmanial activities (Süzgeç-Selçuk et al., 2010).

Even the screening method applied may give rise to different IC_{50} values, such as the resazurin method which has been pointed out as more sensitive than the MTT assay in the assessment of the effect of drugs (Hamid et al., 2004).

Interestingly, the comparison of the antibacterial activity between fresh and dried algae demonstrated that the tested organisms were more sensitive when fresh algae were used, suggesting that the active compounds may be volatile and were lost during the drying process (Tuney et al., 2006). Likewise, the storage conditions of samples may influence the bioactivities obtained through the loss of valuable compounds (Srivastava et al., 2007).

Besides *C. baccata*, *C. barbata* and *C. tamariscifolia*, only two panel presentations focus on the antileishmanial potential of *C. usneoides*, *C. humilis* and *C. nodicaulis*, with similar results in comparison to this work (Bruno de Sousa et al., 2012; 2014).

However, phlorotannins extracted from the brown macroalgae *C. usneoides* collected in Peniche, Portugal, exhibited strong antifungal properties combined with a significantly decrease in the amount of ergosterol, the main sterol of *Leishmania* membrane (Lopes et al., 2013). Thus, the strong antileishmanial activity observed in this

study for the dichloromethane extract of *C. usneoides* could be related to the presence of compounds targeting ergosterol.

The study of the *Cystoseira* extracts here presented are currently undergoing bioguided fractionation studies in an ongoing PhD project. In this context, the most interesting extracts, as the *C. baccata*'s, are being studied for their antileishmanial activity with the ultimate goal of isolating the bioactive compounds.

As referred for halophyte extracts in II-3.2., a careful interpretation of the selectivity indexes of *C. tamariscifolia*, *C. usneoides* and *C. barbata* active extracts may be needed due to the diversity of compounds present in the latter. Fractionation of these extracts may unravel the specificity of the selectivity indexes observed, since synergistic effects between compounds may also account for the activity observed. As discussed earlier, it would be interesting to evaluate if these extracts remain active towards the intracellular model of *Leishmania.* Furthermore, the potential of the non-active extracts of macroalgae, as enhancers of the macrophage activation, could be pursued in further studies as suggested for the halophytic extracts.

3.2.1. Effect of *Cystoseira* extracts on *L. infantum* promastigote morphology

Because the HEX extracts of *C. baccata* and *C. barbata* displayed low cytotoxicity against THP-1 cells, but were still active towards *L. infantum* promastigotes, a microscopic analysis of the their leishmanicidal effects was performed.

Since the HEX extract of *C. baccata* at the maximum concentration tested exhibited mixed morphology of the affected parasites, only the image acquired of the effect of *C. barbata* HEX extract is presented (Figure 19D). For comparison purposes, the HEX extract of *C. nodicaulis*, which did not presented activity, was included (Figure 19C).

At 125 µg mL^{-1}, the extract of *C. barbata* significantly reduced the parasite's viability to 37% and in parallel induced deep morphological changes on promastigote cells, as observed by light microscopy.

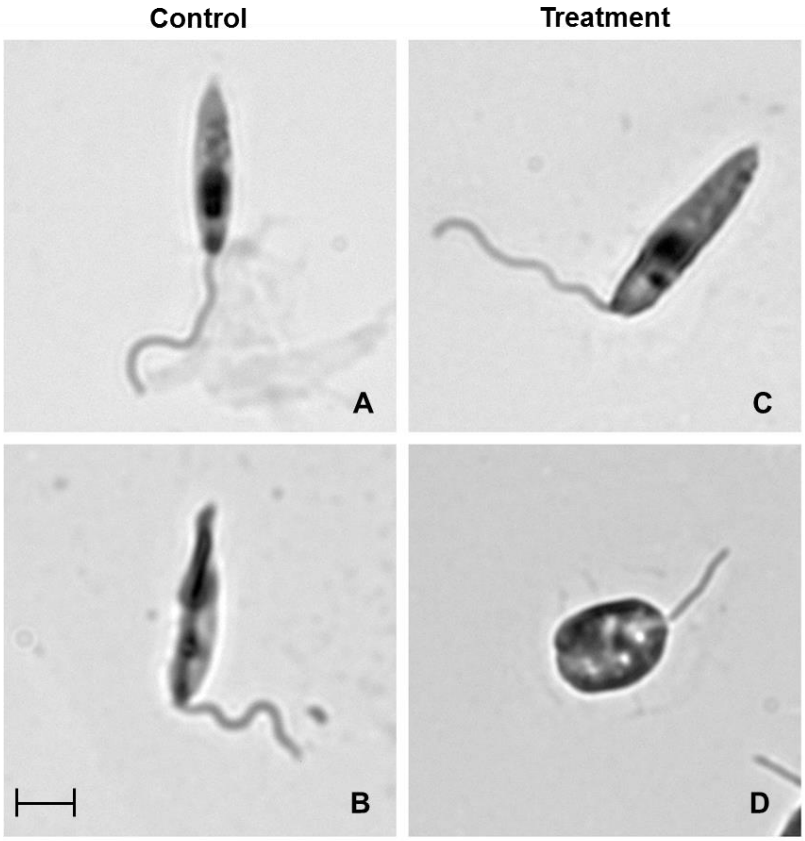

Figure 19 Effect of *C. barbata* and *C. nodicaulis* HEX extracts (125 µg mL^{-1}, 48h) on the morphology of *L. infantum* promastigotes: **A**, Control cells cultured in RPMI medium; **B**, positive control, AmB (IC_{50}); **C**, *C. nodicaulis*; **D**, *C. barbata*. Bright field images, scale bar corresponds to 5 µm.

Control cells have a normal shape and both kinetoplast and nucleus are well distinguished. *C. barbata* induced considerable changes in parasites morphology such as cell shrinkage accompanied with an abnormal round shape, dense cytoplasm and also, some parasites presented loss of the flagella. Moreover, the loss of parasite's motility was evident.

As expected, since *C. nodicaulis* was inactive towards *L. infantum* promastigotes, it did not cause any clear morphological changes or loss of mobility, in comparison with control cells.

The HEX extract of *C. baccata* caused less pronounced but similar morphological changes than the ones induced by *C. barbata* extract (data not shown). Affected parasites had round to oval shape, with short flagella and were not motile. Nevertheless, some parasites exhibiting normal morphology were observed and a few still presented normal

motility. This can be due to the fact that the IC_{50} value of *C. baccata* is higher than that of *C. barbata*.

Similarly, the positive control was tested at its IC_{50} value. The majority of cells presented alterations in the membrane of the cell, which may be due to the fact that AmB interacts with the main sterol, namely ergosterol, present in the *Leishmania* parasites membrane, leading to membrane disruption, as discussed before in I-1.4.

The observed alterations were also reported in *L. tropica* promastigotes treated with propolis samples (Duran et al., 2008; Ozbilge et al., 2010), in *L. amazonensis* promastigotes treated with ethanolic extracts of *Azadirachta indica* (Carneiro et al., 2012) and *L. major* promastigotes treated with *Allium sativum* extract (Khademvatan et al., 2011).

As these are preliminary observations, future work involving bioguided fractionation and ultrastructural analysis of promastigote morphology could unravel the mechanisms of action of the selected fractions or compounds, in combination with other methodologies for this purpose (Neto et al., 2011).

3.3. Evaluation of the effect of the extracts on the amastigote-macrophage model

In the context of this work, the antileishmanial activity of *C. barbata* and *C. baccata* HEX:DCM extracts against intracellular amastigotes of *L. infantum* was evaluated. Similarly, the toxicity of the extracts was assessed towards THP-1 macrophages, to determine the selectivity index.

Samples of these two species were extracted by using a hot soxhlet-based system to increase the yield of bioactive compounds. As mentioned in III-3.2., based on previous results obtained in the MarBiotech group, this extraction methodology was more efficient than the cold centrifugation extraction technique in achieving higher amounts of the compounds of interest (Oliveira et al., 2014).

Having in mind the latter results concerning the promastigotes primary screen, as these two species have presented moderate activity along with lower cytotoxicity to the mammalian macrophages, its activity was tested on the amastigote-macrophage system, using the previously optimized direct counting method (Figure 20).

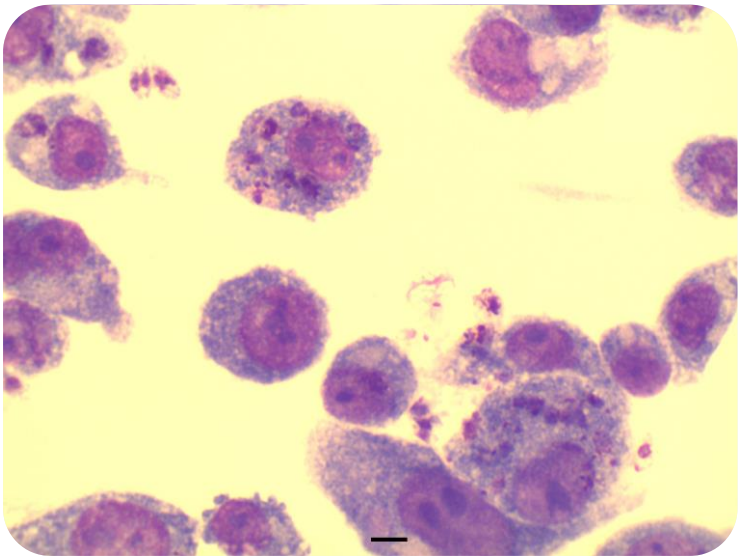

Figure 20 THP-1 infected cells used for the assessment of the activity of *C. baccata* and *C. barbata* extracts by direct counting method previously optimized (Original from Oliveira, M.). Scale bar corresponds to 10 µm.

In contrast with the results observed for promastigotes using cold extracts, the *C. barbata* extract was the most interesting, with an IC_{50} value of 15.1 ± 2.9 µg mL^{-1}, while that of *C. baccata* was less active with an IC_{50} of 50.2 ± 2.6 µg mL^{-1}. As a consequence of the cytotoxicity determined for these extracts, SI of *C. barbata* and *C. baccata* were greater than 8.3 and 2.5, respectively (Table 10).

Table 10 Antileishmanial activity towards intracellular amastigotes (IC_{50}, µg mL^{-1}), cytotoxicity (IC_{50}, µg mL^{-1}) and selectivity indexes (SI) obtained for *C. baccata* and *C. barbata* extracts.

Species	**IC_{50} values** (µg mL^{-1})		**Selectivity index** (SI)
	L. infantum	**THP-1**	
C. baccata	50.2 ± 2.6[a]	>125	>2.5
C. barbata	15.1 ± 2.9[b]	>125	>8.3
Amphotericin B	0.1 ± 0.0[b]	>125	>1250
Miltefosine	0.4 ± 0.1[b]	9.0 ± 0.5	22.5

Values represent the mean ± SEM obtained from three independent experiments with at least two replicates each. * represents the positive controls. Values followed by different letters are significantly different ($p < 0.05$; Tukey's test).

Interestingly, when tested towards the promastigote form, *C. baccata* Hex:DCM extract was more active than a similar extract of *C. barbata* (IC_{50}, 30.6 ± 2.8 and 74.7 ± 6.0 µg mL^{-1}, respectively; Oliveira et al. 2014). However, towards the amastigote-macrophage system, *C. barbata* was clearly the most interesting extract, with a selectivity index greater than 8.3. In fact, miltefosine was not selective towards the promastigotes (SI = 0.5) either; conversely, its selectivity towards amastigotes was high (SI = 22.5). These results highlight the relevance of the *in vitro* model used for efficient research of natural products with antileishmanial activity. Additionally, the presence of the macrophage may influence the effectivity of the extract to affect the intracellular parasites as discussed in I-3.1.

Although the SI of the positive control AmB is 156 fold greater than that of *C. barbata*, the SI of miltefosine is only 3 fold greater. However, and as discussed above in III-3.1., considering that an extract is a mixture of compounds, the fractionation of the extract and further isolation of the bioactive compound may lead to increased selectivity.

It would be interesting to elucidate the mechanisms of action of the selected active fractions or compound(s) as well as the morphological and ultrastructural changes induced in parasites in future lines of research. Moreover, other mammalian cellular lines should be tested to confirm the cytotoxic values obtained, including the peritoneal macrophages, as a useful indication for additional in *vivo* studies.

To our knowledge, this is the first report of *C. barbata* and *C. baccata* effect on the *in vitro* infection of THP-1 macrophages with *L. infantum* parasites.

In summary, and despite the low antileishmanial activities displayed by the majority of macroalgae in this study, some of the species tested may be considered as interesting sources of bioactive compounds against *L. infantum* parasites, namely *C. baccata* and *C. barbata*.

Chapter IV - Conclusions

Current available drugs for human leishmaniasis treatment exhibits several disadvantages such as high costs, toxicity, long-term periods of parental administration for some drugs, variation in intrinsic sensitivity between *Leishmania* species and development of drug resistances (Croft & Coombs, 2003; Singh et al., 2012).

In the last decades, marine natural products have been investigated for their antileishmanial compounds and some promising results have been achieved. However, the drug screening for natural products with antileishmanial properties exhibits several difficulties mainly due to the lack of standardization of the screening methodologies and parasite *in vitro* models used.

In this work the mammalian cell infection model was established using metacyclic promastigotes of *L .infantum* and differentiated THP-1 cells as host cells. This model was used for optimization of the direct counting method conditions for further testing of the most interesting extracts towards the clinical relevant stage of the parasite.

The obvious drawbacks of the direct counting method were the main encouragement for our efforts to optimize a methodology that would enable the screening of large numbers of extracts by observing their effect on the amastigote-macrophage system. Thus, the parasite rescue assay, a methodology involving the lysis of infected cells with consequent liberation of the amastigotes, was, and still is, a possible avenue of research in order to implement a laboratory procedure that could attain that goal. An encouraging result is that the optimal lysis conditions were established. The fluorescence measurements, however, still needd to be fully optimized in the near future. To address the optimization of this final step, both time of incubation and concentration of resazurin must be studied until conditions optimal for assessing the effect of the extracts are established. In spite of its challenges and pitfalls, the unquestionable advantages of this method should be not ignored. Hence, if developed further, it might prevent the discarding of promising extracts upon evaluation of their activity towards the extracellular form of the parasite, namely promastigotes.

Furthermore, the antileishmanial activity of halophyte and macroalgae extracts were investigated, and the results indicate that some of these organisms may present compounds with antileishmanial activity. *S. rubra* and *I. crithmoides* DCM extracts as well as the HEX and DCM extracts of the macroalgae *C. tamariscifolia* and the HEX extract of *C. usneoides* were the most active towards *L. infantum* promastigotes, yet

poorly selective. The HEX extract of the macroalgae *C. baccata* displayed the higher selectivity index of all the extracts tested. Although the SI were not very interesting, further investigation on these active species should be reconsidered, having in mind the possibility of distinct compounds being responsible for the antileishmanial and cytotoxic activities observed, and also that the activity may be specific to each parasite stage. Thus, more interesting results are possible to be achieved.

Despite the fact that the majority of extracts were inactive towards the promastigote form of *L. infantum*, it should not be concluded that these species do not contain bioactive compounds. In future work, other solvents, more efficient extraction methods or even other halophyte parts should be used in an attempt to extract the compounds of interest. Moreover, *C. baccata* and *C. barbata* HEX:DCM hot extracts showed higher selectivity against *Leishmania* intracellular amastigotes, reinforcing the importance of both the extraction technique and the *in vitro* model used. *C. barbata* displayed the most promising selectivity (SI = 8.3) in comparison to the positive controls, even though the bioactive(s) compound(s) are mixed with other inactive molecules within the extracts used.

Last but not least, these results may encourage additional research on bioactive compounds from *C. barbata*. Thus, the search for novel bioactive compounds should be pursued not only via bioguided fractionation, but also by using peritoneal macrophages as the mammalian model. This would yield more reliable results regarding cytotoxicity, before assessing the *in vivo* activity of the active fractions or compound(s).

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